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Role and regulation of neutral sphingomyelinase-2 in response to DNA damage

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Abstract of the Dissertation

Role and regulation of neutral sphingomyelinase-2 in response to DNA damage

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Neutral sphingomylinase-2 (nSMase2) is a ceramide-generating enzyme that has been implicated in growth arrest, apoptosis and exosome secretion. In this thesis, we show that doxorubicin induces a dose-dependent induction of nSMase2 mRNA and protein levels concomitant with an increase in neutral sphingomyelinase activity and ceramide levels. This induction appears to be specific to agents that inhibit topoisomerase I as well as those that produce single stranded DNA breaks. Upregulation of nSMase2 was dependent on ATR, Chk1 and p53, thus placing it downstream of the DNA damage pathway. Evidenced by knockdown studies, nSMase2 appears to play a protective function as its knockdown sensitizes cells to doxorubicin. Elucidating that mechanism, nSMase2 appears to regulate a transient S phase checkpoint through its prolongation. Its knockdown results in an earlier recovery from S phase arrest. Consistent with its function as a checkpoint regulator, we find that nSMase2 localizes to the nucleus following doxorubicin treatment and is housed in distinct subnuclear granules termed nuclear speckles. Interestingly, a specific lipid, C18:1 ceramide appears to be the major product of nSMase2 regulating its biology. Overexpression of nuclear targeted bacterial sphingomyelinase or exogenous media

supplementation with oleate rescues the cells from doxorubicin-induced cell death. Using a phosphoproteomics approach, we determined possible phosphorylation targets of nSMase2. This revealed the cohesin complex as a major downstream target of nSMase2. Taken together, these studies demonstrate the essential role of nSMase2 in DNA damage-induced S phase arrest through the regulation of the cohesion complex

Dedication Page

To Mom,

To Dad,

To Lama,

To Antonella

It's the simplest things that make life worthwhile

Achraf

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List of Abbreviations

Act D	Actinomycin D
APL	Anionic phospholipids
Ara-C	Arabinofuranosylcytosine
AS	All Star negative control siRNA
ATRA	All-Trans Retinoic Acid
bCDase	Bacterial ceramidase
BrdU	Bromodeoxyuridine
bSMase	Bacterial sphingomyelinase
C1P	Ceramide-1-phosphate
CERS	Ceramide synthase
CERT	Ceramide transfer protein
COPD	Chronic obstructive pulmonary disease
Dox	Doxorubicin
dsDNA	Double stranded DNA
EED	Embryonic ectoderm development
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ET-1	Endothelin-1
FAN	Factor associated with neutral sphingomyelinase
GCS	Glucosylceramide synthase
GSH	Glutathione
hnRNA	Heteronuclear RNA
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
IR	Ionizing radiation
MMP	Metallomatrix protease
MOMP	Mitochondrial outer membrane permealization
NAC	N-acetylcysteine

NGF	Nerve growth factor
NSD	Neutral sphingomyelinase activation domain
nSMase1	Neutral sphingomyelinase-1
nSMase2	Neutral sphingomyelinase-2
PP2A	Protein phosphatase 2A
PS	Phosphatidylserine
Rb	Retinoblastoma
RDS	Radioresistant DNA synthesis
ROS	Reactive oxygene species
S1P	Sphingosine-1-phosphate
SK1	Sphingosine kinase 1
SK2	Sphingosine kinase 2
SM	Sphingomyelin
SMS1	Sphingomyelin synthase 1
SMS2	Sphingomyelin synthase 2
SPT	Serine palmitoyl transferase
ssDNA	Single stranded DNA
TNF-α	Tumor necrosis factor alpha
TOPBP1	Topoisomerase binding protein 1
TRAIL	TNF-related apoptosis ligand
TSS	Transcription start site
UTR	Untranslated region
UV	Ultra-violet
Veh	Vehicle

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3. **Shamseddine AA**, Newcomb BJ, Obeid LM, Hannun YA. Dasatinib inhibits doxorubicin-induced invasion at sublethal doses. *In preparation.*
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5. **Shamseddine AA**, Faraj W, Mukherji D, El Majzoub N, Khalife M, Soubra A, Shamseddine AI. (2010) Unusually young age distribution of primary hepatic leiomyosarcoma: case series and review of the adult literature. *World journal of surgical oncology* **8**, 56

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5. Zgheib NK, **Shamseddine AA**, Geryes E, Tfayli A, Bazarbachi A, Salem Z, Shamseddine AI, Taher A, El-Saghir NS. (2013) Genetic polymorphisms of CYP2E1, GST, and NAT2 enzymes are not associated with risk of breast cancer in a sample of Lebanese women. *Mutation research.* **747**, 40-47
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Chapter 1

Overview of bioactive sphingolipids and the roles and regulation of neutral sphingomyelinase-2

1.1 Introduction

Sphingolipids are a family of bioactive lipids implicated in numerous biological processes. Initially, the name of the "sphingosine" backbone was forged by a German chemist, J.L.W. Thudichum in reference to the many mysteries the study of this lipid presented. In Greek mythology, the sphinx guarded the entrance to the city of Thebes and only granted entry to travelers who answered its riddle.

The central hub of the sphingolipid pathway is the bioactive lipid ceramide. Ceramide can be generated through different pathways (1,2). The *de novo* generation of sphingolipids start with the condensation of serine and palmitoyl CoA to generate 3-ketodihydrosphingosine in a reaction catalyzed by a family of enzymes, the serine palmitoyl transferases (SPTs) (3). SPTs also have the ability to utilize alanine and glycine instead of serine in a pathway that eventually forms deoxysphingolipids, a terminal class of lipids that is unable to exit the sphingolipid pathway due to the absence of enzymes that metabolize it (4,5). Following formation of 3-ketodihydrosphingosine, it is reduced to dihydrosphingosine which subsequently gets N-acylated to dihydroceramide by ceramide synthases (CerS) (1,2). There are 6 mammalian ceramide synthases characterized to date and they differ by their fatty acyl chain length specificity. As such, this generates a myriad of ceramides, each differing in the chain length of their fatty acid (6,7). Finally dihydroceramide reductase inserts a double bond at the 4,5 position of the sphingosine backbone to form ceramide (8).

Subsequently, ceramide can have multiple fates. Addition of a phosphocholine headgroup by sphingomyelin synthases results in the formation of sphingomyelin (SM), a major component of cell membranes (9). Ceramide can be also glycated to form more complex glycosphingolipids

(2). Finally, a major pathway studied is ceramide conversion to sphingosine in a deacylation step catalyzed by a class of enzymes termed ceramidases. Five of these enzymes have been cloned and are classified based on their pH optima of their activity into acid, neutral and alkaline (3 alkaline ceramidases exist) (10-12). It should be noted that neutral and alkaline ceramidases have reverse activities and can convert sphingosine back to ceramide (13). Alternatively, this reaction can be performed also by ceramide synthases. This pathway is termed the "salvage pathway" as it rescues ceramide back and prevents its exit from the sphingolipid pathway (1,2). Additionally, sphingosine turn can be phosphorylated on its hydroxyl head group to yield sphingosine-1-phosphate (S1P) (14). S1P can be broken down by a lyase into hexadecenal and ethanolamine phosphate which constitutes the point of exit of this pathway (15).

1.2 Sphingolipid metabolism is highly compartmentalized

The *de novo* production of ceramide constitutes the only point of entry into the sphingolipid pathway. The main reactions occur in the endoplasmic reticulum (ER) and once ceramide is produced, it is transferred to the Golgi apparatus where it can form complex sphingolipids, namely SM by the addition of a phosphocholine headgroup, glycosphingolipids through the addition of a sugar headgroup or ceramide-1-phosphate (C1P) through phosphorylation (1,2). SM synthesis in the Golgi is thought to occur through sphingomyelin synthase 1 (SMS1) and sphingomyelin synthase 2 (SMS2) (16,17). It is believed that the ER to Golgi transport occurs in a non-vesicular fashion through the ceramide transfer protein (CERT) (18). After synthesis in the Golgi, complex sphingolipids are distributed in a vesicular fashion to the different cellular compartments (19).

In the plasma membrane, SM is localized primarily on the outer leaflet. However, a small pool of SM is localized in the inner leaflet of the plasma membrane and is used as a substrate for

the action of neutral sphingomyelinase-2 (nSMase2) to form ceramide (20). The reverse reaction of conversion of ceramide to sphingomyelin can occur at the plasma membrane and is catalyzed by SMS2 (21). Furthermore, neutral ceramidase is also present in the plasma membrane, albeit on the outer leaflet (22). It is unclear whether the substrate of neutral ceramidase comes from ceramide targeted to the plasma membrane in a vesicular fashion or from the flipping of nSMase2-generated ceramide from the inner leaflet of the plasma membrane. Sphingosine Kinase 1 (SK1) is also present in the plasma membrane and localizes there in response to different stimuli to produced S1P (23-26).

The bulk of SM degradation occurs in the lysosome through the action of the lysosomal form of acid sphingomyelinase. Defective activity of this enzyme is the genetic basis for Niemann-Pick disease that is characterized by failure of growth and psychomotor regression in children as well as progressive worsening of respiratory functions (27,28). Ceramide generated in the lysosome can serve as a substrate for the generation of sphingosine through acid ceramidase. The deficiency of this enzyme is the genetic basis of Farber disease which is characterized by widespread abnormalities and eventual death due to pulmonary complications in children (29). To note, there is no characterized presence of any sphingosine kinases in the lysosome. As such, it is assumed that lysosomal sphingolipids must be transported into other organelles to exit the sphingolipid pathway.

Sphingomyelin presence in the nucleus has been reported and mapped to different subnuclear localization (30). The intricacy of how sphingomyelin gets to the nucleus is still unclear. Two distinct possibilities arise. The first is a vesicular transport from the Golgi and the second is its nuclear generation from ceramide that is transported from the ER. The second possibility is more probable as sphingomyelin synthase activity was detected in nuclear enriched

fractions (31). Sphingomyelin can be degraded in the nucleus by neutral sphingomyelinase-1 (nSMase1) which was shown to localize to the nucleus (32). Furthermore, sphingosine can be generated through the action of nuclear ceramidases as well as S1P through sphingosine kinase 2 (SK2) (33,34) .

Mitochondrial sphingolipids have also been detected. Ceramide synthase activity has been detected in partially purified mitochondrial-enriched fractions suggesting the potential for either *de novo* synthesis of ceramide or its salvage generation to occur in the mitochondria (35). Furthermore, a recently cloned mitochondrial-associated neutral sphingomyelinase was found to localize to the outer mitochondrial membrane (36,37). No ceramidases or sphingosine kinases were identified in the mitochondria yet.

Taken together, these results point to a very specific compartmentalization of sphingolipid metabolism. Functionally, as we are beginning to gain the molecular tools to study the enzymes, it would be of utmost importance to assign specific functions to organelle-specific sphingolipids and to be able to manipulate those organelle-specific pools. This could have major implications in understanding signaling processes, and using sphingolipids as potential targets for disease treatments.

1.3 Biological functions of ceramide

Ceramide is a bioactive lipid that has been implicated in numerous cellular processes. Below is an overview of the major biologies where ceramide generation was found to play a major role.

1.3.1 Apoptosis

Extensive literature has described the effect of ceramide on cell death. The first report by Obeid et al. described an effect of exogenous C2-ceramide treatments on induction of DNA fragmentation and programed cell death in leukemia cells (38). This was selective to ceramide as dihydroceramide was unable to induce the same biology (39). Furthermore, exogenous addition of sphingomyelinase to fibrosarcoma cells was demonstrated to induce DNA damage and apoptosis (40). At the time, that was the first report of an endogenously generated ceramide mediating the apoptotic function. Subsequently many stimuli were shown to induce ceramide generation and apoptosis and these included tumor necrosis factor-alpha (TNF-α), Fas and ionizing radiation (1). Most of these effects were thought to occur though the hydrolysis of sphingomyelin either by neutral or acid sphingomyelinase activities. However, the first reports of the involvement of *de novo* ceramide synthesis in mediating apoptosis came from studies on daunorubicin. These studies demonstrated a role for daunorubicin-generated ceramide in mediating cell death that was inhibited by the ceramide synthase inhibitor, Fumonisin B1 (41). Following these initial studies, many of the subsequent literature elaborated on different stimuli that induce ceramide generation and cell death in multiple cell lines.

The precise mechanism of how ceramide induces cell death involves activation of both the intrinsic and the extrinsic pathway of apoptosis. The intrinsic pathway is characterized by mitochondrial outer membrane permeabilisation (MOMP) and cytochrome c release. Interestingly, MOMP directly correlates with the level of ceramide in the outer mitochondrial membrane (42). However, some studies suggest that ceramide is not sufficient to induce MOMP but rather its synergism and activation of BAX is required for apoptosis (43-45). Ceramide can also induce MOMP through caspase 2 and caspase 8 activation. This can occur following GSK3 beta activation in response to induction of cathepsins, downregulation of akt or activation of protein phosphatase

2A (PP2A) (46-49). Finally, ceramide has also been shown to induce the mitochondrial translocation of PKCδ which induces cytochrome c release and caspase 9 activation (50).

The extrinsic pathway of apoptosis is mediated mainly through receptor activation of endogenous caspases and cell death. Many receptors engage the extrinsic pathway and these include TNF receptors and the TNF-related apoptotic ligand (TRAIL) receptors. It is widely believed that these receptors generate ceramide at the plasma membrane and localize them to ceramide-enriched membrane platforms (51,52). Much of the understanding of ceramide function comes from cancer cell resistance to the extrinsic pathway of apoptosis and the modulation of that resistance by sphingolipid and ceramide generation. For instance, resistance to TRAIL-induced cell death is overcome by expression of Ceramide synthase 6 (CerS6) (53). Furthermore, TNF receptor 1 promotes the formation of ceramide at the plasma membrane through nSMase2 activation (more on that below). Failure of that activation is associated with resistance to TNF-induced apoptosis of breast tumors (53). A last possible way of ceramide activating the extrinsic pathway is through downregulation of the FLICE protein, an endogenous inhibitor of caspase 8. This has been shown to occur in glioblastoma and prostate cancer (54,55).

1.3.2 Growth arrest

The initial characterization of ceramide function came from studies in the Hannun lab on serum-starved Molt-4 leukemia cells. It was noticed that, following serum withdrawal, cells arrested in G0/G1 phase with accumulation of ceramide from sphingomyelin hydrolysis. Conversely, exogenous addition of C6 ceramide recapitulated the same phenotype (56). Subsequent studies suggested that this effect was possibly mediated through the retinoblastoma gene (Rb) product (57). Studies on the involvement of ceramide in G0/G1 arrest demonstrated also

the involvement of nSMase2 in mediating confluence induced growth arrest (58). This was attributed to the dephosphorylation of beta catenin in a PP1cgamma-dependent manner suggesting a signaling mechanism mediating this effect (59).

More importantly, some studies on ceramide function in growth arrest concentrated on a potential role of ceramide in regulating cell cycle checkpoints. This is of particular interest as cell cycle regulators represent one of the major areas where focus is intense to develop novel chemotherapeutics to treat malignant tumors. The most developed of these studies suggest the major function of ceramide to occur at the G1/S transition through 2 signaling avenues. The first involved the activation of p21 and the dephosphorylation of Rb (60) and this can occur both in a p53-dependent and in a p53-independent manner (61).The second involves the inhibition of the G1 cyclin-dependent kinase CDK2 (62).Interestingly, a recent studies suggested a third mechanism by which ceramide can control the G1/S transition. In response to All Trans Retinoic Acid (ATRA), nSMase2 is activated and appears to mediate a G1 arrest that is dependent on the dephosphorylation of S6 kinase with no effect on either Rb or p21 (63).

The data about ceramide regulating the G2/M transition. To date only a couple of studies reported effect of ceramide on G2/M. The first suggest that, in rhabdomyosarcoma cells, ceramide induces a G2 arrest that is concomitant with increased p21 and downregulation of cyclin D (64). The other suggested that ceramide suppresses surviving which controls the G2/M transition (65). As such, the distinct mechanisms of how ceramide controls the G2/M checkpoint as well as its application to endogenous situations of ceramide upregulation remains unclear. To note, there has not been to date studies that pointed to roles of ceramide in either the S phase checkpoint or in the mitotic spindle checkpoint.

1.3.3 Senscence

Cellular senescence is a process where cells lose the ability to proliferate. It is different from quiescence in that it is thought to be irreversible and associated with aging. A lot of studies have implicated sphingolipids in aging and some of these will be discussed further below. However, the focus of this section is on the involvement of ceramide in cellular senescence. The first observation implicating ceramide came from the Obeid lab where ceramide was found to be increased in senescent human diploid fibroblast due to neutral sphingomyelinase activity (66,67). This was also associated with dephosphorylation of Rb and inhibition of AP-1 activation (66,68). Subsequent studies showed induction of B-galactosidase by exogenous ceramide addition in these fibroblasts (69), and in human umbilical vain endothelial cells (70). Furthermore, exogenous treatment of ceramide in these cell lines was also found to dephosphorylate Rb commonly seen with ceramide-induced cellular senescence. Recent evidence suggests that metformin, a drug used in the treatment of diabetes, can reverse ceramide-induced senescence in C2C12 myoblasts (71). However, it is unclear if the action of metformin is in the same pathway or in a parallel pathway to ceramide. Finally, and while most of these studies implicated a neutral-sphingomyelinase generated ceramide in the induction of senescence, a recent study suggest that the lack of CERT, and thereby the lack of transfer of ceramide from the ER to the Golgi resulted in premature senescence in mouse embryonic fibroblasts (72).

1.3.4 Autophagy

Autophagy is a cellular protective mechanism aimed at the degradation of unused cellular metabolites and organelles to preserve cellular energy. However, cancer cells use this mechanism to promote their survival and as such, understanding the processes that regulate this phenomenon

can lead to its efficacious targeting. Ceramide has long been implicated in autophagy (73-75). Many mechanisms have been proposed, the most convincing of which show regulation of the mTOR pathway and nutrient uptake (76). Interestingly enough, there seems to be a consensus that regulation of autophagy seems to occur through the long-chain dihydroceramides (73,77). The drug fenretinide has been used extensively in these studies due to its dual action as an activator of SPTs as well as an inhibitor of dihydroceramide desaturase which results in the accumulation of dihydroceramide. Briefly, feretinide was shown to induce autophagy in breast, pancreatic and cervival cancer cell lines (78-80).

1.4 Introduction to neutral sphingomyelinase-2

Sphingomyelin hydrolysis is catalyzed by a class of enzymes referred to as sphingomyelinases (SMases) to generate ceramide, a bioactive lipid involved in diverse cellular processes (1,2). SMases are classified based on their pH optima of activity into acid, neutral, and alkaline subtypes. Of the four different mammalian neutral SMases that have been identified; neutral sphingomyelinase-2 (nSMase2) appears to be the predominant nSMase in cellular systems, physiologies, and pathologies (81-84). This review will focus on the roles and regulation of this enzyme emphasizing recent findings implicating nSMase2 in disease processes.

1.5 Characterization and regulation of nSMase2

1.5.1 Cloning

Cloning of nSMase2 revealed it as part of a protein superfamily that hydrolyzes phosphodiester linkages and requiring Mg^{2+} for activity. Both the human and mouse nSMase2 gene (SMPD3) encode for a 655 amino acid protein with a molecular mass of 71 kDa that contains an

N-terminus with 2 hydrophobic segments and a C-terminus consisting of the catalytic site (85). The mouse and human versions are very similar and share 90% sequence identity.

1.5.2 Basic biochemical properties

NSMase2 specifically hydrolyzes the phosphocholine-headgroup from sphingomyelin and does not exhibit Phospholipase c-type activity against phosphatidylcholine, lyso-phosphatidylcholine, platelet activating factor or lyso-platelet activating factor. Neutral pH and divalent cations (Mg^{2+} or Mn^{2+}) are required for activity, while phosphatidylserine (PS) and unsaturated fatty acids stimulate enzymatic activity in vitro (85). A later study found that sphingosylphosphocholine, the deacylated form of SM, can be hydrolyzed under detergent-free conditions by nSMase2 (86).

1.5.3 Structural features

To date, there has not been a crystal structure reported for nSMase2. Therefore, our current understanding of the structure and mechanism of nSMase2 is based on investigations of related bacterial SMases. Three different structures of bacterial neutral SMases from the pathogenic organisms *Bacillus cereus*, *Listeria ivanovii*, and *Staphylococcus aureus* has confirmed neutral SMases belong to the DNase I type protein superfamily, which also includes inositol phosphatases (87-90). NSMase2 shares relatively low sequence identity with the bacterial versions but most likely shares a similar protein fold and catalytic mechanism for SM hydrolysis (87,90). Studies by Tani et al showed that the enzyme harbors two hydrophobic loops at the amino terminus rather than the sequence-predicted transmembrane segments (20).

1.5.4 Cellular localization

After initially cloning nSMase2, Hoffman et al. showed by antibody staining that nSMase2 localized to the Golgi apparatus in both PC12 and SH-SY5Y cells (85). Subsequently, overexpressed nSMase2 was shown to localize to the plasma membrane (PM) in the confluence phase of MCF7 cells and in primary hepatocytes (58,91). Building on these findings, Tani et al. found that nSMase2 localized to the inner leaflet of the PM in confluent MCF7 cells and was palmitoylated at two Cysteine clusters (20,92). The first cluster encompasses Cys53, Cys54 and Cys59 that are present between the 2 hydrophobic segments of the N-terminus, and the second cluster encompasses Cys395 and Cys396, which are present at the beginning of the catalytic site (92). Studies with cycloheximide in subconfluent MCF7 cells demonstrated that the Golgi localization of overexpressed subconfluent nSMase2 is the result of two protein pools: newly synthesized protein and a second that recycles back from the PM through the endosomal system (93).

1.5.5 Regulation by anionic phospholipids

Prior to the cloning of the different isoforms of neutral SMases, anionic phospholipids (APLs) were found to increase neutral sphingomyelinase activity in rat hepatomas. Delipidated membranes treated with Triton X-100 recovered NSMase activity only upon addition of PS, phosphatidic acid, phosphatidylinositol but not the neutral lipids phosphatidylcholine or phosphatidylethanolamine (94). These findings were later confirmed in a purified membrane bound neutral sphingomyelinase from rat brain (95). Following the cloning of nSMase2, biochemical characterization showed that the enzyme has a catalytic pH optimum of 7.5, with a strong stimulation by PS or cardiolipin and a requirement for either Mg^{2+} or Mn^{2+} for activity (85,96). The activity of the previously cloned neutral SMase1 was shown to be APL-independent (97), thus identifying nSMase2 as the previously characterized magnesium dependent neutral

SMase that was activated by APLs. A detailed mechanistic study found that APLs bind to the N-terminus of nSMase2 at two distinct positively charged sites. The first site binds both PS and phosphatidic acid and required R33 and the amino acids 45-48 (KRQR). The second site selectively bound PS and required R92 and R93 (98). Biochemically, APL binding was found to affect both the substrate affinity (K_m) and rate of hydrolysis (V_{max}) of nSMase2. Mutation of the APL binding sites altered the localization to the ER. In yeast strains lacking the nSMase2 homolog Isc1, the wild type protein corrected sensitivity to hydroxyurea, while the mutant protein was unable to do so (84) [Fig1].

1.5.6 Regulation by phosphorylation

NSMase2 was originally identified as a phosphoprotein in human bronchial epithelial and A549 cells with phosphorylation occurring basally on serine residues (99). The serine/threonince phosphatase Calcineurin, (also known also as protein phosphatase 2B) was found to bind a PQIKIY motif between the N-terminus and the C-terminus to dephosphorylate nSMase2 (99). The phosphorylation of nSMase2 under H_2O_2 stimulation in A549 cells occurs on 5 conserved serine residues: S173, S209, S291, S294 and S301, which are located near the calcineurin binding site. Alanine point mutants lacking the phosphorylation sites are not phosphorylated by H_2O_2 and are not activated in response to oxidative stress. In addition protein phosphorylation at these sites regulated the stability of nSMase2 in this cellular model (100). In addition, some of nSMase2 functional effects have been found to be dependent on protein kinases. For instance, p38 and PKCδ regulate the translocation of nSMase2 to the plasma membrane in response to TNF (101). Furthermore PKCδ role in this translocation is independent of effects on the activation of nSMase2 in response to TNF (102). However, it is not known if these kinases phosphorylate nSMase2 directly or affect nSMase2 function through signaling pathways.

1.5.7 Transcriptional regulation of nSMase2

Recent insights into stimuli activating nSMase2 shed light on some aspects of its transcriptional regulation. The chemotherapeutics Daunorubicin and Camptothecin were found to induce nSMase2 transcription in MCF-7 breast cancer cells and K562 leukemia cells via activation of the putative promoter by the tansciption factors Sp1 and Sp3. The region of the promoter necessary for this activation was mapped to -147 bp upstream of exon 1 (103). ATRA was also found to induce transcriptional activation of nSMase2 via the same mechanism (104).

1.5.8 Tools for the study of nSMase2: inhibitors and mouse models

Initial studies of NSMase activity relied heavily on the natural occurring inhibitors Scyphostatin and Manumycin A that were originally identified from fungi and bacteria respectively (105,106). These inhibitors had a broad NSMase inhibitory activity with no selectivity towards different NSMase isoforms. In addition, Scyphostatin had inhibited acid SMase activity but with a higher IC_{50} (107,108). A high throughput screen for small molecule inhibitors of nSMase2 identified GW4869 as a selective nSMase2 inhibitor with an IC_{50} of 1 μM. GW4869 is a non-competitive nSMase2 inhibitor and its mechanism of action is thought to be through interference with APL activation of the enzyme (108,109). Since its identification, GW4869 has been the most commonly employed pharmacological tool to study nSMase2 function in cellular and animal models.

Mice models to study nSMase2 function were characterized at the beginning of the 21st century. Stoffel et al. generated the first $SMPD3^{-/-}$ mouse and showed that it has decreased neutral SMase activity in many liver and brain while the $SMPD3^{-/-}$ $SMPD2^{-/-}$ double knock out mouse, which deletes both nSMase1 and nSMase2, abolished all neutral SMase activity (110). The

fragilitas osseum (fro/fro) mouse model, which was originally identified in a chemical screen for bone defects, was shown by Aubin et al. to have a catalytically inactive nSMase2 due to deletion of the C-terminal 33 residues and has since been used as an additional model to study nSMase2 function. The original study reported the fro/fro mouse retained residual NSMase activity to almost 10% of the wild type (111). More recent reports have found overexpression of the fro/fro mutation in a cellular model did not increase nSMase2 activity. This is more consistent with the deletion of two catalytically conserved residues, D638 and H639, in the fro/fro mutatation (82,84) and it was speculated that residual nSMase activity in the fro/fro mouse is due to the presence of other nSMase isoforms.

1.6 NSMase2 as a regulator of biological functions

NSMase2 has been implicated in many physiological processes as well as disease pathologies. In this section, we will review the most important biological functions relating to nSMase2 and highlight potential avenues for future research.

1.6.1 Inflammation

Response to TNF-α

Activation of neutral sphingomyelinase in response to the cytokine TNF-α was initially observed in the HL-60 cell line. In these leukemia cells, TNF-α induces a rapid SM hydrolysis, peaking at 1 hour concomitant with an increase in ceramide and cellular differentiation into monocytes (112). The initial discovery was replicated in a cell-free system (113) and it required the TNF-α receptor-1 (TNFR-1), commonly known as the p55 receptor (114-117). Mechanistically, a distinct region of the p55 receptor spanning amino acids 309-319 was found to mediate the increase in NSMase activity and was named the Neutral Sphingomyelinase Activation

Domain (NSD) (116). The 9 amino acid stretch of the NSD binds a protein termed Factor Associated with Neutral sphingomyelinase (FAN) that influences NSMase activity in response to TNF-α (117,118). FAN is a protein of the WD-40 family and contains 5 WD repeats, which are common scaffolding motifs involved in coordinating multi-protein complex assemblies. The N-terminus of FAN is critical for NSMase activity as a truncation of FAN, which contained only the WD repeats, inhibits NSMase activation following TNF-α in a dominant negative fashion (117,118). Following that, Receptor for Activated C-Kinase 1 (RACK1) was identified as a FAN interaction partner using a yeast two hybrid system. The interaction between FAN and RACK1 was verified both in vitro and in cells and is dependent on the C-terminal WD repeats of both proteins. RACK1 was also shown to modulate N-SMase activation following TNF-α treatment (119). Subsequently, a yeast-two hybrid interaction mapping suggested the polycomb protein embryonic ectoderm development (EED) as a binding partner of nSMase2 via its C-terminus. Further investigation showed that EED also binds to RACK1 via the same region that interacts with nSMase2, and rapidly translocates from the nucleus to the PM after TNF-α treatment. The proposed model is one where EED interacts with RACK-1 and nSMase2 and brings them together in a multi-protein complex with TNFR1 through FAN, hence establishing EED as a critical modulator of nSMase2 activation following TNF-α (120).

In addition to the elucidation of the molecular partners, other investigations have focused on cellular factors that influence TNF-α activation of nSMase2. Liu et al. reported that the levels of glutathione (GSH), which can inhibit neutral SMase activity, are critical for TNF-a activation. Their model suggests that baseline inhibition of nSMase2 activity is due to physiologic levels of GSH and TNF-a treatment activates nSMase2 by decreasing levels of GSH (121). Further studies in A549 lung cells demonstrated that TNF-α induces a translocation of nSMase2 from the Golgi

to the PM that was largely dependent on p38 MAPK (101) and Protein Kinase C delta (PKC-δ) (102). In vascular smooth muscle cells and fibroblasts, TNF-α activation of nSMase2 was shown to require the activation of the metallomatrix protease 2 (MMP2) from its inactive precursor form MT1-MMP2 by proteolysis mediated by Furin (122).

Functionally, the activation of nSMase2 has implications in the inflammatory response of TNF-α. Studies in HeLa cells showed that nSMase2 acts on the PI3K/Akt pathway to activate endothelial nitric oxide synthase (eNOS) (123). In HUVEC cells, this effect required the conversion of ceramide into S1P, by the action of SK1 and eNOS activation regulated the expression of adhesion molecules like E-selection and VCAM-1 (124). Clarke et al. expanded on this theme and demonstrated that in A549 cells, nSMase2 inhibition attenuated TNF-α stimulation of VCAM-1 and ICAM-1 (101). In addition to the vascular effects of TNF-α, nSMase2 mediates some of the effects of TNF-α on the neural system. TNF-α stimulates nSMase2-dependent ceramide production in primary hippocampal neurons that modulates synaptic plasticity through trafficking of NR1 subunits of N-methyl-D-aspartate receptors to lipid rafts (125). Another report suggests that activation of nSMase2 in primary cortical neurons precedes reactive oxygen species (ROS) formation and damage to neurons (126). It is interesting to note that the TNF-α derived vascular effects seem to require the sequential action of both nSMase2 and SK1, while in contrast the neuronal effects of TNF-α is dependent only on nSMase2 activation. This suggests that nSMase2-derived ceramide can have bioactive action by itself in response to TNF-α or can be metabolized into other lipids to induce differential responses [Fig2].

Response to Interleukin-1 beta (IL-1β)

The role of nSMase2 in inflammation has been studied extensively in response to another cytokine, IL-1β. IL-1β is a pro-inflammatory cytokine found to be secreted by inflammosomes, which is an early pathogenic feature of many liver and other organ systems diseases (127). IL-1β was first reported to induce NSMase activity in rat hepatocytes (128). Subsequent studies in murine cortices demonstrated that this activation requires the IL1-Receptor 1 (IL1-R1) based on the observation that IL1-R1 KO mice failed to increase NSMase activity after IL-1β stimulation (129). The major isoform activated by IL-1β was demonstrated to be nSMase2 as overexpression of nSMase2 in IL-1β treated HepG2 cells enhanced phosphorylation of Janus Kinase (JNK) acutely (91). In the same system, nSMase2 overexpression suppressed phosphorylation and increased the stability of the Interleukin-1 receptor-associated kinase (IRAK-1) through activation of the protein phosphatase 2A (PP2A) family (91). Further studies identified the phosphatase isoform involved as PP2Ac and showed that nSMase-2-dependent dephosphorylation of IRAK-1 by PP2Ac delayed IRAK-1 ubiquitination, which led to its stabilization and amplification of the IL-1β response by nSMase2 (130).

Functionally, the role of nSMase2 in the IL-1β response was elucidated through aging models. Aged hepatocytes display increased sensitivity to IL-1β stimulation that is translated via higher JNK phosphorylation as well as higher IRAK-1 stability (131). This is due to an increase in nSMase2 activity in aged hepatocytes that is dependent on decreased levels of GSH (131). Attenuation of nSMase2 levels by siRNA or by shRNA decreased IRAK-1 stability and JNK phosphorylation to restore a normal response to IL-1β in aged hepatocytes (132). Moreover in normal rat hepatocytes and in HEK 293 cells, nSMase2 was shown to stimulate post-transcriptional FOXO1, accumulation in the nucleus through its JNK/ERK activation in response to IL-1β (133). In glial cells, nSMase2 was shown to be important in IL-1β dependent Src

activation that mediates IL-6 secretion (134). Taken together, these results place nSMase2 as an attractive drug target in inflammatory diseases of the liver and other organ systems.

Response to Interferon gamma (IFN-γ)

IFN-γ is another cytokine that was studied in the induction of nSMase2 activity. IFN-γ was shown to initially increase NSMase activity with acute stimulations in HL-60 cells (112). Subsequently, nSMase2 was demonstrated to be the major NSMase activated in response to IFN-γ and induced translocation of PKC-δ to the Golgi in HeLa cells in a JAK1/2 dependent manner (135). In RAW264.7 macrophages IFN-γ activates an nSMase2 → PP2A → Akt/GSK → Nitric oxide pathway (136); while in rat vascular smooth muscle cells, NSMase inhibition by 3-O-methyl sphingomyelin prevented IFN-γ-mediated nitric oxide production. This is due to the inhibition of translocation of p65 subunit of NF-k κB to the nucleus and hence failure of the NF- κB signaling cascade to activate iNOS at the mRNA level (137).

Involvement in phagocytosis

NSMase2 has a described role in phagocytes, which are typical cells of acute inflammation that engulf bacterial cells or other small cells. Polymorphonuclear cells, of which the phagocytic neutrophils constitute the major portion, display a plasma membrane NSMase activity that increases during phagocytosis (138). Inhibition of nSMase2 by GW4869 causes loss of directional motility and migration in these cells (139). Finally, peptidoglycan, a major component of bacterial cell wall, was shown to induce nSMase2 activity in human macrophages that leads to the activation of p38 MAPK and NF-κB to increase cyclooxygenase-2 expression (140).

1.6.2 Involvement in pulmonary pathophysiology

Recent findings have demonstrated a functional regulation of nSMase2 in response to lung insults and in certain lung diseases. Initially, Castillo et al. reported that H_2O_2 stimulation and ROS formation activates nSMase2 to mediate apoptosis in human airway epithelium, while glutathione (GSH) pre-treatment prevents apoptosis (141). Building on this in a pathophysiological context, it was found that cigarette smoking, a potent ROS-generating lung insult, increased NSMase activity acutely in immortalized human airway epithelium leading to apoptosis (142). GSH pre-treatment or siRNA to nSMase2 were found to inhibit apoptosis, concordant with the previous study. Consistent with the cellular studies, mouse models of cigarette smoke exposure showed an accumulation of ceramide in lungs of mice 1-3 weeks after exposure, causing apoptosis (143). There was a simultaneous increase in nSMase2 protein expression in both bronchial epithelium and alveolar septae of mice that was prevented by pretreatment with the ROS scavenger N-acetylcysteine (NAC) (143). One major consequence of chronic smoking is the development of chronic obstructive pulmonary disease (COPD), a clinical entity comprising two distinct pathologies, namely emphysema and chronic bronchitis. Conflicting results have been reported on the involvement of nSMase2 in the pathophysiology of emphysema. Poirier et al. showed increased morphological changes that resemble emphysematous changes in lungs of fro/fro mice in the absence of any insult (144). These changes included alveolar distention and increased lung compliance by pulmonary functional testing, which suggests that lack of nSMase2 activity in the fro/fro mice mediates emphysematous changes (144). In contrast, Filosto et al. reported increased nSMase2 activity in the alveolar space of emphysematous patients (143). Tibboel et al. observed in an elastase-treatment model of emphysema that there was an nSMase2-independent increase in ceramide levels in broncheo-alveolar lavage fluid at day 2 to 5 post-treatment (145). NSMase2 has also been implicated in hypoxic pulmonary vasoconstriction, which is a physiologic pulmonary

smooth muscle vasoconstriction in response to poor perfusion in order to shunt the blood supply to better-oxygenated alveoli (146). Hypoxia induces a GW4869-inhibited acute ceramide increase and pulmonary arterial smooth muscle contraction (146). Further elucidation of the mechanism revealed that nSMase2 activation in rat pulmonary arteries in response to hypoxia generated ROS and was prevented by blockage of the mitochondrial NADPH oxidase (147). Finally, Lin et al. suggested a role for nSMase2 and sphingosine kinase in antagonizing anti-apoptotic properties of lipopolysaccharide during acute lung injury, which improved lung function and survival in these patients (148). Taken together, these studies suggest a profound role of nSMase2 in modulation of lung responses to pathologies.

1.6.3 Involvement in circulatory and cardiac pathophysiology

nSMase2 in circulatory conditions

NSMase2 has been implicated in the development of hypertension. Initial findings pointed to activation of a membrane NSMase in response to acute changes in vascular pressure associated with NSMase-dependent ERK1/2 and MEK1/2 phosphorylation (149,150). Applying this finding to animal models, young and old rat aortas and mesenteric vessels were studied. Old aortas lost acetylcholine mediated vascular relaxation, which was associated with inhibitory phosphorylation on eNOS (151). NSMase activity was also increased, and GW4869 treatment improved the vasomotor action of aged vessels (151,152). Furthermore, the inhibitory effect on eNOS was recapitulated in a mouse model of ER stress that showed increased nSMase2 activity associated with inactivation of eNOS. NSMase2 overexpression mimicked ER stress and resulted in decreased NO production, a potent vasodilator (153,154). These effects suggest that activation of nSMase2 is associated with a vasoconstrictor phenotype. In that context, the effect of the

physiologic vasoconstrictors endothelin-1 (ET-1) on nSMase2 was studied. ET-1 activates nSMase2 to induce VCAM-1 expression in rat small mesenteric artery (155). Probing the system further, the effect of dietary Mg^{2+} deficiency was studied. Mg^{2+} deficiency has been demonstrated experimentally and is clinically associated with hypertension. In rats, a Mg^{2+} deficient diet for 21 days increased NSMase activity in both aortic smooth muscle and the left ventricle (156). This was reversed by supplementing the diet with magnesium and resulted in alterations of telomerase activity that correlated with NSMase inhibition (156,157). Taken together, this presents nSMase2 as a potential target for pharmacological inhibition in clinical hypertension.

Studies have implicated nSMase2 in various other circulatory pathologies. Transplant vasculopathy is a major cause of transplant rejection and is mediated by anti-HLA antibodies of the host. Anti-HLA activates nSMase2 via MMP2 in smooth muscle cells and produces a mitogenic signaling that leads to thickening of the intima of the vessels (158). The MMP2 →nSMase2 → mitogenic activation cascade is also found in endothelial ECV304 cells in response to urokinase. Here, integrin$\alpha_5\beta_3$ activation of MMP2 is a critical step for nSMase2 activation (159). Finally, nSMase2 activity has implications in circulatory changes in dyslipidemia. Reports suggest that ApoC-1 enriched HDL particles, which are HDL particles with limited peripheral absorption, leads to an nSMase2-dependent apoptosis, when exogenously added to aortic smooth muscle cells (160).

nSMase2 in cardiac conditions

The literature on nSMase2 in cardiac pathologies is not thoroughly developed, but seems to suggest that activation of nSMase2 occurs in cardiac conditions and is, at least partly, responsible for cytotoxic outcomes. Early studies reported that following ischemia, reperfusion

caused acute activation of a Mg^{2+}-dependent NSMase in rat cardiac myocytes (161), which was prevented by pre-treatment with NAC, and NSMase-derived ceramide activated the JNK pathway (161). Furthermore, post-myocardial infarction rat hearts displayed reduced GSH and its repletion using NAC resulted in inhibition of a nSMase2/Bcl-2/caspase3 axis and decreased oxidative stress resulting improved left ventricular function (162). Another report suggested that obese type II diabetic patients have increased serum NSMase activity but that serum ceramide levels were unaffected (163).

1.6.4 Involvement in neurobiology and neuropathology.

Response to neurotrophins

Neurotrophins are a family of four mammalian proteins that promote neuronal survival and function through 2 classes of receptor mediated signaling; the p75 neurotrophin receptor, which is a common receptor for all neurotrophins and the Tropomyosin-receptor kinase (Trk) receptors that selectively bind specific neurotrophins. Initial studies demonstrated activation of the sphingomyelin cycle following neurotrophin treatment in T9 gliomas and PC12 cells (164,165). Application of the neurotrophin Nerve Growth Factor (NGF) to cultured hippocampal neurons produces ceramide via nSMase2 activation to engage apoptosis (166). NSMase2 activation requires the p75 receptor and increases phosphorylation of JNK (166). This effect is recapitulated in adult motor neurons as NGF-induced apoptosis is blocked by the nSMase inhibitors manumycin A and GW4869 (167). p75 activation of nSMase2 also increases action potentials in capsaicin sensitive sensory neurons through increased current in tetrodoxin resistant sodium channels and suppression of a delayed rectifier outward potassium current (168). Other data suggest that nSMase2-derived ceramide may also act upstream of p75 receptors. Peng et al. showed that penta-

acetyl geniposide induces NSMase activity in C6 gliomas with ceramide generation that is inhibited by GW4869 (169). NGF and p75 are upregulated in that setting in an nSMase2-dependent manner (169). Moreover, the role of nSMase2-derived ceramide in apoptosis/survival in response to neurotrophins has recently been clarified. Candalija et al. reported a protective, cell survival-promoting role of nSMase2 in response to the neurotrophin brain-derived neutrophic factor in granule neurons and NGF in PC12 cells (170). However, unlike previous studies, the increase in cell survival stemmed from activation of the Trk receptors (170). This leads to a possible model where activation of the p75 low affinity receptor in response to NGF promotes an apoptotic nSMase2-dependent signal, while neurotrophin activation of the Trk receptors promotes a pro-survival nSMase2-dependent signal.

Activation of neuronal death

A body of literature suggests the involvement of nSMase2-derived ceramide in neuronal death. Ethanol activates nSMase2 to produce glial cell death and signals through the ERK, JNK and p38 MAPK pathways in astrocytes (171). On the other hand H_2O_2 causes apoptotic cell death via activation of nSMase2 in primary oligodendrocyte, a finding that sheds light on one of the contributing factors to the exacerbation of multiple sclerosis, a demyelinating disease involving oligodendrocytes (172). Kainic acid-induced status epilepticus increases pro-apototic Bax and decreases anti-apoptotic Bcl-2 levels simultaneous with nSMase2 activation (173). Finally, oxidized phosphatidylcholine increases nSMase2 at the mRNA level in rat oligodendrocytes and leads to the activation of caspase-3 and caspase-8 (174).

Alzheimer's disease

There is growing interest in the involvement of nSMase2 in the pathogenesis of Alzheimer's disease. Initial reports showed that $A\beta_{25-35}$ peptide upregulated NSMase activity in rat primary astrocytes and neurons causing cell death (175,176). Further studies suggested that $A\beta_{1-42}$ and $A\beta_{1-40}$ peptides activated nSMase2 to negatively control gamma secretase activity (177). Recently, inhibition of phospholipase D2 was shown to abrogate Aβ-nSMase2 activation in SH-SY5Y cells (178). The importance of exosomal secretion of Aβ peptide mediated by nSMase2 is beginning to gain appreciation (179-181). It is thought that exosomes released from microglia prevent Aβ oligomerization, through unknown mechanisms and that the absence of exosomes leads to a soluble Aβ peptide that produces neuronal death (181).

Other neurological functions

Effects of nSMase2 on normal synaptic regulation was studied in models of dopamine uptake where it was shown that nSMase2 inhibition or downregulation by siRNA decreased dopamine uptake in PC12 cells (182) with Hsp60 acting as an interaction partner that negatively regulates nSMase2 in that system (183). This paved the way to more in depth studies of involvement of nSMase2 in dopaminergic pathologies such as Parkinson's disease. Physiologically, nSMase2 inhibition by GW4869 delayed formation of spatial reference memory in mice without affecting episodic-like memory; the likely mechanism is through the modification of the subunit composition of N-methyl-D-aspartate receptors (184). Also, a role for nSMase2 was found in inflammation following ischemia/reperfusion injuries in the brain. Astrocytes, but not neurons, displayed increased nSMase2 activity following ischemia/reperfusion with nSMase2-dependent generation of the pro-inflammatory cytokines TNF-α, Interleukin-1 and Interleukin-6 (185). Finally, a potential role for nSMase2-derived ceramide was recently described in embryonic stem cells and embryonic stem cell-derived neural progenitors. In these cells, ceramide induce

ciliogenesis, a critical step in differentiation that was inhibited by GW4869 and fumonisin B1, an inhibitor of ceramide synthase. NSMase2-derived ceramide prevented the activation of histone deacetylase-6, Aurora A, and sequestered protein kinase C into apicolateral domains preventing its activation (186). Taken together, these findings suggest a profound role of nSMase2 in neuronal pathologies. This will pave way for research understanding more in depth mechanisms of neuronal regulation by nSMase2, but also for the development of specific inhibitors of nSMase2 as novel strategies of treatment of neurological diseases.

1.6.5 NSMase2-mediated release of exosomes

Recently, a body of literature emerged involving nSMase2 in exosome release through a non-canonical pathway independent of ESCRT protein complexes. Exosomes are 40-100 nm vesicles that are released from the cell by the fusion of multivesicular endosomes to the plasma membrane. The resulting secreted exosome carries cellular contents such as protein, RNA, and lipids and serves to influence biological functions of neighboring cells through the transfer of these contents (187). In tumors, nSMase2 was initially shown to mediate exosomal release from HEK293 containing mir16 and mir146 (188). The functional significance of this was demonstrated by showing that blocking nSMase-2 mediated exosomal release from 4T1 breast xenografts reduced lung metastasis through perturbation of endothelial function (189). Isolated exosomes from these tumors increased HUVEC tube formation and migration through exosomal transfer of mir-210 (189). However, this function seems to be tumor specific as another reports suggested that in androgen-resistant prostatic cell line PC-3, nSMase2 does not play a role in exosome release and instead suggested a role of glycolipids in that release (190).

The role of nSMase2 in Alzheimer's disease is mediated at least partly through exosomes. Primary astrocytes from murine cortices treated with $A\beta_{25-35}$ or $A\beta_{1-42}$ died along with induction of PAR-4, production of ceramide and caspase3 activation (180). The PAR4 along with the ceramide was found to localize to exosomes, and that finding was not present in the fro/fro mice (180). Treating wild-type mice and 5XFAD Alzheimer's disease mouse model with GW4869 resulted in lower exosomes in the brain, with the 5XFAD mice displaying lower whole brain concentration of $A\beta_{1-42}$ (179). Finally, nSMase2 mediated exosomal transfer was found to be important in antigen presentation. Antigen presenting cells secrete nSMase2-dependent exosomes, enriched in small RNA species, which regulate antigen presentation (191).

1.6.6 Role of nSMase2 in cancer

The role of sphingolipids in cancer is a subject that has gained attention recently. While the involvement of nSMase2 in chemotherapeutic-mediated cytotoxicity has been the subject of intense examination, the contribution of nSMase2 in the pathogenesis and perpetuation of human tumors is less well understood (192). In leukemia, mutations of the nSMase2 gene, SMPD3, were identified in 5% of acute myeloid leukemia and 6% of acute lymphoid leukemia. Some of these mutations perturbed the stability or localization of the enzyme (193). In breast cancer, SMPD3 was one of 55 genes with more than 4 fold upregulation in tumor vasculature of luminal A type breast cancer versus control matched breast tissue (194). Moreover, the metastasis of 4T1 murine breast tumors was found to be dependent on nSMase2-mediated exosome transfer of miR-210 (189). Moreover, a recent study identified nSMase2 as a possible tumor suppressor in hepatocellular carcinoma as genome-wide analysis revealed hypermethylation of the gene (SMPD3) in human samples. Further experiments demonstrated that overexpression of nSMase2 in these cells slowed growth while its downregulation promoted cell invasion and migration (195).

From a therapeutic standpoint, the early hints at a role for nSMase2 mediating effects from tumor therapy came from studies that found stimulation of NSMase activity following 1-beta-D-Arabinofuranosylcytosine (Ara-C) peaking at 30 minutes with increase in ceramide in HL-60 cells (196). A similar effect was seen with ionizing radiation (IR) (197). Erythromyeloblastic cell lines that are resistant to IR fail to activate NSMase and do not undergo apoptosis (198) while c-kit activation, which confers resistant to IR, prevents nSMase2 activation (194,199). Following that, daunorubicin, etoposide, paclitaxel and ara-C were found to activate nSMase2 in different cell lines (50,192,200) via a PKC-dependent mechanism, to mediate the cytotoxic effects of these chemotherapeutics (50,200). Compounds with anti-tumor properties that are not used in clinics have also been studied. Polyphenols (t-PER and QUER) activate nSMase2 (201), while nSMase2-dependent apoptosis was described in K562 and MOLT-4 in response to Withanolide D (202), and in K562 and HT29 in response to Protopanaxadiol (203). Finally, compounds derivative of p53 reactivation and induction of massive apoptosis (PRIMA-1), possess nSMase2-mediated cytotoxic properties against lung cells (204). The cytotoxic effect of nSMase2 activation can also mediate some side effects of chemotherapeutics. Gentamycin-induced ototoxicity is prevented by GW4869, as pre-treatment with this inhibitor decreased outer hair loss in organs of Corti explants (205).

1.6.7 Role of nSMase2 in cell death

One of the earliest described biological consequences of ceramide formation is cell death. NSMase2 activation to engage cell death machinery has been described both in acute (minutes to a few hours) and in prolonged (>24 hours) stimulations. Early on NSMase-derived ceramide action on cell death was defined in TNF-α mediated apoptosis. Activation of NSMase was shown initially in response to TNF-α (112), before Obeid et al. demonstrated that TNF-α induced apoptosis and

exogenous addition of C2 ceramide mimicked that effect (38), and similar results were shown using exogenous sphingomyelinases (40). Further characterization was done using bacterial sphingomyelinase as a tool mimicking NSMase activation in cells. Zhang et al. showed that exogenous application of *Bacillus Cereus* cloned sphingomyelinase resulted in cell death with activation of PARP (206). Other members of the TNF receptor family such as FAS have been also shown to mediate activation of NSMase to cause cellular apoptosis. Agonist of Fas induced accumulation of ceramide with an increase in membrane bound SMase activity and DNA fragmentation in Fas sensitive cells, a finding that was mimicked with exogenous addition of ceramide in Fas resistant cells (207,208). Many stumuli were subsequently shown to activate NSMase. In WEHI231 cells, cross-linking of IgM increases membrane sphingomyelinase activity and ceramide production causing apoptosis, preventable by overexpression of Bcl-xL (209). PC12 cells activate sphingomyelinase in response to hypoxia to produce apoptosis peaking at 24 hours. Similar to WEHI231s, this is blocked by overexpression of the anti-apoptotic Bcl2 (210), or by pre-treatment with NAC or GSH (211). This observation was consolidated in C6 glioma cells where Bcl-2 overexpression prevented etoposide-induced nSMase2 activation (212). Work by Chipuk et al. studied how NSMase-derived ceramide cooperate with Bax and Bcl-2 to promote apoptosis. Isolated mitochondria where found to be resistant to release of cytochrome c following cleaved caspase 8 (C8-BID) treatment. A restoration of the sensitivity occurred only when the microsomal fraction (containing NSMase activity) was added to the mitochondrial fraction, and the resensitization was blocked by GW4869 (213). While the use of GW4869 in that study suggests the involvement of nSMase2 as the NSMase isoform responsible for the ceramide generation, the lack of data on microsomal nSMase2 interacting with mitochondria coupled with the unavailability of data relating to GW4869 inhibition of the recently cloned mitochondrial-associated NSMase

lead to the conclusion that further studies are required to determine the NSMase isoform responsible for the biology described.

The effector pathway of apoptotic cell death is through activation of caspases. The role of caspase activation in nSMase2-mediated cell death has been reported with conflicting results. While most studies suggest caspase activation follows the activation of nSMase2 (214), some define caspase activation as a pre-requisite for nSMase2 activation. For example, inhibition of caspase-3 prevented nSMase2 activation in HL-60 cells following sodium nitroprusside treatment and addition of recombinant caspase-3 activates NSMase activity in a cell free system (215).

One disease process in which nSMase2-mediated apoptosis seems to be relevant is diabetes mellitus (DM). One of the proposed mechanisms of pathogenesis of DM is ER stress and the activation of the unfolded protein response. Initial findings showed that basal nSMase2 was activated in Akita mice, a mouse model of ER stress (216). Subsequently pancreatic β islets treated with thapsigargin were shown to undergo apoptosis with activation of nSMase2 at the message level (217) and that nSMase2 upregulation is mediated by iPLA2 (217,218). Finally, phosphatidylinositol ether lipid analogs (PIAs) induce secretion of pro-apoptotic factors in non-exosomes nanovesicles in an nSMase2-dependent manner (219).

1.6.8 Role of nSMase2 in cell differentiation and growth arrest

The role of NSMase activity in cell differentiation was examined prior to the cloning of the different isoforms of NSMase. Vitamin D-induced cell differentiation in HL60 leukemia cells was accompanied by activation of a neutral sphingomyelinase and ceramide generation, while the addition of an exogenous SMase potentiated the differentiation effect of subthreshold Vitamin D (220) with ceramide being identified as the sphingolipid responsible for that effect (221). The

effect of NSMase activation on HL60 monocytic differentiation was also shown in response to TNF (112) and the later characterization of nSMase2 as the major NSMase activated by TNF suggest this effect is nSMase2 specific. Later studies expanded on the role of NSMase in cell differentiation, with the effect of neurotrophins on the activation of nSMase2 (refer to section C) and the potential role of that in neuronal differentiation being the most characterized of these functions.

The study of nSMase2 in modulation of the cell cycle has gained momentum recently with the discovery of stimuli that activate nSMase2 to produce growth arrest. While ceramide has been described to produce a growth arrest phenotype, among its many biological functions, studies of nSMase2-derived ceramide has lagged behind (2). MCF7 breast cancer cells overexpressing nSMase2 have a similar growth phenotype to control cells in the exponential phase. However, upon serum starvation they get retained in G0/G1 to a greater extent than cells overexpressing control plasmid. Moreover, cell confluence upregulates nSMase2 to arrest cells in G0/G1 with hypophosphorylation of the retinoblastoma protein and induction of p21. Downregulation of nSMase2 by siRNA bypasses this phenotype (58). Later studies demonstrated nSMase2-dependent dephosphorylation of beta catenin on T41/S45 via activation of PPC1γ in confluent cells (59). Another stimulus that induces nSMase2-dependent growth arrest is ATRA. ATRA induces activation of nSMase2 at the transcriptional level in cells with functional retinoic acid receptor-α to arrest them in G1 phase of cell cycle (222). The mechanism is thought to be through the dephosphorylation of S6K, independent of PP2A (63).

H. Role of nSMase2 in post-natal growth and bone development

Stoffel et al. initially described the severe growth retardation in the SMPD3$^{-/-}$ mice starting at embryonic day 14 and accompanied with hypoplasia of all organs except the brain. They also described delayed bone ossification present in the mice (110). Later, studies on the fro/fro mice showed a reduction in mineralized tissue in long and flat bones. The hypertrophic chondrocytes in long bones persist and do not undergo either apoptosis or mineralization in long bones. The localized expression of SMPD3 corrected this phenotype (223). The mechanism by which nSMase2 acts to control chondrocyte maturation and mineralization was further studied to describe two key pathways. The first pertained to hyaluronan synthesis, an essential process in normal bone maturation. Fro/fro fibroblasts were found to have increased hyaluronan with upregulation of hyaluronan synthase-2 (HAS-2) (224). In ATDC5 cells, a common cellular model of chondrocyte maturation, HAS-2 was found to be downregulated during maturation and siRNA to nSMase2 prevented HAS-2 downregulation (225). Another mechanism thought to partially contribute to the phenotype is the effect on Pi3K/Akt pathway. Fro/fro mice were found to have higher levels of phosphor-Akt and phosphp-S6K and knockdown of nSMase2 in ATDC5 cells during differentiation further potentiated this increase (224,225).

1.7 Conclusion:

A number of tools developed over the past two decades have enlarged our understanding of the regulation, enzymology and biologic functions of nSMase2. However, many questions still remain [Fig.3]. The determination of a crystal structure of this enzyme may answer basic questions regarding the enzymology and the mechanism of activation. There is a need to understand cellular mechanisms of regulation of nSMase2 and the specific mechanisms by which ceramide and other downstream lipid metabolites mediate the various actions of this critical enzyme. More in depth

studies are also needed to transition biological findings into clinical studies with the anticipation that this enzyme could be a drug target for the treatment of several disease processes.

Chapter 2

Overview of the DNA damage response and the S phase checkpoint

2.1 Introduction

Most eukaryotic cells are in a quiescent, non-dividing state, commonly referred to as G_0. In response to proliferative stimuli, some cells can exit G_0 and commit to division by entering the cell cycle. The cell cycle refers to the ensemble of cellular processes that starts with the exit from the quiescent state ends up with the formation of two daughter cells. It is comprised mainly of 2 main phases, the first is the interphase where the cell prepares itself for division by ensuring proper duplication of biomaterials, synthesis of nutrients and increase in size; and the second is mitosis, in which actual cell division occur to yield two daughter cells (226). The interphase is composed itself of 3 phases, G1, S and G2 (226). The G1 phase is a predominantly growth stage in which cells synthesize mRNA and proteins required for DNA synthesis (227). Once complete, cells enter S phase where duplication of the genome occurs (228). Following exit from S phase, cells go into G2, a non-essential phase that is absent in some organisms (Xenopus) and where the cell readies itself for mitosis (229). Finally, mitosis is the phase in which chromosomes as well as proteins get segregated in two different cells to complete the cell cycle (230).

The control of cell division is an evolutionary mechanism essential in maintaining chromosomal integrity and in preventing mutations to the DNA, genetic instability and tumorigenesis. Central to this control are molecular circuitries commonly referred to as checkpoints. Each checkpoint serves as a control to ensure the proper completion of the phases. For instance, the G1 checkpoint is pre-replicative and integrates many cellular inputs that evaluate the fitness of the cell to undergo mitosis (227). P53 and its transcriptional target p21 play an essential role in the establishment of the G1 checkpoint (227). On the other hand, the S phase checkpoint controls faithful replication of chromosomes. Together with the G2 checkpoint, they regulate cellular responses to genotoxic stress in order to ensure genome integrity. It is believed

that both of these checkpoints are essential to arrest the cell cycle to allow the cell to repair any damage occurred. Interestingly, both appear to be controlled by similar signaling proteins, namely the DNA damage proteins ATM, ATR, Chk1 and Chk2 (228,229). P53 appears to have a secondary role in the establishment of S and G2 checkpoints as some studies reported that it is necessary but not essential for these checkpoints. Finally, the mitotic spindle checkpoint is an intra-M phase checkpoint that controls proper alignment of chromosomes and their separation in order to prevent aneuploidy (230). In this section, we will focus mostly on the S phase checkpoint. These excellent reviews provide more information about the G1, G2 and the spindle checkpoint (227,229,230).

2.2 p53: a tumor suppressor and a central hub in the DNA damage pathway

P53 is a major tumor suppressor gene that is mutated in many sporadic human malignancies. Its mutations are the genetic basis for Li-Fraumeni syndrome, a disease characterized by an inheritance of a mutant *TP53* allele and in which patients have predispositions to early-onset tumors including breast carcinomas, leukemias and sarcomas (231). The tumor suppressor function of p53 is also described in murine models, as p53 knockout mice have high rates of spontaneous tumors, including thymic lymphomas (232). Typically, a sporadic or inherited *TP53* mutation is not enough to trigger tumo genesis and is followed by a loss of heterozygocity which results in complete p53 deficiency (233). This participates in the initiation or progression of cancer as tumors that lack p53 have genetic instability and increased invasiveness (234-236). These properties are conferred by both the loss of wild-type tumor suppressive p53 functions and the oncogenic gain of function of p53 mutants (237,238). P53 is a transcription factor and a cellular stress sensor that controls the expression of both genes and miRNAs to trigger both transient and permanent cell arrest, and apoptosis in response to DNA damage, hypoxia, oxidative stress, and nutrient starvation (239-241). These conditions can occur in normal tissues and therefore are

relevant *in vivo* triggers for p53 activation. P53 can also have non-transcriptional functions of which the most important is the activation of caspase-independent apoptosis (242). While the canonical thinking implicated the growth arrest and apoptotic functions of p53 in tumor suppression, recent evidence suggests p53 regulates additional processes including cellular metabolism, stem cell function, metastasis, as well as cell-cell communication. All of these functions might contribute to the tumor suppressive function of p53 (240).

Under normal conditions, p53 is bound to Mdm2, an E3 ubiquitin ligase that mediate its degradation (241). Upon genotoxic stress, the activation of the DNA damage pathway causes phosphorylations on both Mdm2 and p53 in an ATM-dependent manner, which prevent their association (241). As such, p53 gets induced and tetramerize to bind to promoter elements via its DNA binding domain (241). The majority of p53 mutations localize to the DNA binding domain, suggesting its importance in tumor suppression (243). Transactivation mutants of p53 (mutants that inhibit its non-transcriptional function) maintain its tumor suppressive function, confirming that the transcriptional functions of p53 are crucial for its tumor suppressive role (244,245). Transcriptionally, p53 tetramers bind to specific response elements composed of two half-sites with the nucleotide sequences RRRCWWGYYY (R = purine, W = A or T and Y = pyrimidine) separated by a spacer of 0-13 nucleotides (246). Genetic studies as well as recent genomic analyses have identified a growing list of p53-regulated genes and their study will be essential in deciphering p53 biologies (247,248)

Acetylation of p53 is one of the topics where growing understanding of its mechanism and implications is becoming apparent. Importantly, acetylation of p53 increases its stability, its transcriptional affinity to promoters and is required in response to DNA damage to mediate the checkpoint as well as the apoptotic functions of p53 (249-251). Lysine acetylation of p53 appears

to be very redundant as mutations of each lysine residue seems to be compensated. However, mutations of the eight major acetylation residues in human p53, the 8KR mutant (K120, 164, 370, 372, 373, 381, 382, 386) renders p53 inert (250).

In terms of mechanism as well as functionality, one can distinguish between the N-terminal acetylation and the C-terminal acetylation. The C-terminal acetylation of p53 is thought to occur through p300 and CBP to mediate an "open" conformation of p53 that enhances its transcriptional activity (252). Depending on the site modified, the acetylation is associated with change in p53 DNA binding affinity as well as particular recruitment of cofactors. For example, p53 acetylation at both K382 and K320 promotes recruitment of p300CBP and TRRAP to the p21 promoter following DNA damage (253).

N-terminal acetylation comprises the acetylation of both K120 and K164. The implications of K164 acetylation is much less known than the K120 acetylation. K120 is located in the DNA binding domain of p53 and plays a critical role in p53 mediated apoptosis, as well as it tumor suppressive function and is mutated in many different types of cancer (254,255). The enzymes that acetylate the C-terminus are different from those that acetylate K120. Most of the research have focused on the acetylation of K120 by three histone acetyltransferases, the MYST family, Tip60 and MOF (254). K120 acetylation of p53 by Tip60 is associated with the selective induction of apoptosis rather than cell cycle arrest (254). This acetylation is required for p53 binding to both the Bax and PUMA promoter (254) while it does not bind to the p21 promoter.

The "guardian" of the genome model was the first that attempted to explain the tumor suppressive function of p53. Under that model, DNA damage induces a p53-dependent G1 arrest, allowing the cells to repair the genome in order to prevent propagation of harmful mutations (256). This occurs through transcriptional activation of the *Cdkn1a* gene that produces the p21 protein,

among others (257,258). Interestingly, p21 knockout mice do not have increased propensity to developing tumors, possibly due to the fact that other p53-regulated cell cycle genes can compensate (259). Two knock-in *TP53* mice provided evidence for the big role of the cell cycle arrest in the tumor suppressive functions of p53. The first is the $Trp53^{R172P}$ and the second is the Trp^{E177R}. Both have defective apoptotic functions but retain partial cell cycle arrest after ionizing radiation that correlates with some p21 induction (260,261). Both mice are resistant to development of the early onset T cell lymphomas characteristic of *TP53* null mice. However, they are ultimately prone to development of late-onset non-T cell lymphomas and sarcomas than wild-type. The ability of these mice to extend tumor latency suggest that the ability of p53 to induce cell cycle arrest is crucial for tumor suppression. Another component of the "guardian of the genome" model is the ability of p53 to stimulate DNA repair following damage. P53 is able to stimulate various DNA repair mechanisms, including nucleotide excision repair, base excision repair and non-homologous end joining (262-264). This occurs through activation of many DNA repair genes, of which the most famous is the Fanconi anemia, complementing group C (*Fancc*) (232). Mice lacking these repair genes are prone to tumors, establishing that the repair function of p53 is important in its tumor suppression (265).

Studies have provided evidence for the activation of the DNA damage response in tumors leading to a p53-centric hypothesis in which oncogene-driven hyperproliferative signals induces aberrant replication and DNA double stranded breaks, resulting in the activation of the DNA damage response and p53-mediated apoptosis or senescence (266-268). However mouse models mimicking the acute activation of the DNA damage response have shown that it is dispensable for p53-mediated tumor suppression (269). For example, one of these studies utilized a p53 fused to an estrogen receptor which permits control of p53 expression through addition of tamoxifen.

However, inducing p53 in this method did not protect from radiation-induced lymphomagenesis, similar to p53 null mice (269). Importantly though, these studies do not exclude complete the role of DNA damage in p53 tumor suppressive function as most of th'em have been done using acute high doses of damaging agents. Tumor cells *in vivo* are likely to encounter more chronic, low-level stress that might promote tumor suppression by p53 utilizing DNA damage pathways.

Finally, the role of the classical tumor suppressive functions of p53 came into question recently with the data that came out from the $p53^{3KR}$ mice. These mice have the acetylation mutations K117R, K161R and K164R (only two of these residues are present in the human gene). Thymocytes and mouse embryonic fibroblasts from these mice fail to undergo apoptosis and cell cycle arrest in response to genotoxic stress, and they cannot induce neither the p21 gene nor the pro-apoptotic genes controlled by p53 such as PUMA (270). However they retain the ability to induce metabolic genes such as glutaminase 2 (*gls2*), *tigar* and *gpx1 (270)*. As such, it still retains the ability to suppress glucose uptake, restrain glycolysis and inhibit ROS accumulation. These triple mutant mice however remain able to retain the tumor suppressive functions of p53 as they do not form tumors like the null mice (270). These surprising findings suggest that p53 tumor suppressive functions do not rely on cell cycle arrest and apoptosis and it might depend on other metabolic functions of p53

2.3 The S phase checkpoint

2.3.1 Overview

Due to its transient nature, the S phase checkpoint was commonly believed to be a "minor" checkpoint. Most of the characterization of the signaling cascade that result in the checkpoint was

established in the fission yeast, *Saccaromyces Cerevisiae*. These organisms presented an ideal model for the study of cell cycle control as they are easily manipulated both genetically and through assays that follow chromosomal fate. The first characterization of the S phase checkpoint came in *S. pombe* where a replication control was required to enter mitosis and that was dependent on the kinases mec1 (ATR) and mec2 (also rad53 and in humans Chk2) (271). Further characterization in the *S. Cerevisiae* model revealed that these kinases are essential for the S phase checkpoint (Paulovich and Hartwell 1995; Zhou and Elledge 2000). In mammals, however, and while ATR still maintains its essential function, Chk1 appears to be the major kinase that has homologous function to Rad 53.

2.3.2 ATR, a major orchestrator of the S phase checkpoint

The S phase checkpoint gets activated under conditions of DNA damage or nucleotide depletion (272). It is thought that the activating lesion is a ssDNA break that either occur through a DNA damage insult or through DNA unwinding by the MCM helicase uncoupled from DNA synthesis in the case of nucleotide depletion (273). Following that, replication protein A binds the ssDNA which results in the activation of the ATR binding protein ATRIP and subsequently ATR (274,275). There are no known differences between the phenotypes that result from loss of ATR or loss of ATRIP in any organism suggesting that ATRIP should be considered an obligate subunit of ATR. This whole series of events is important for correct ATR localization to sites of ssDNA breaks (274), but not for its activation. In fact, ATR signaling is dependent on the colocalization of the ATR-ATRIP complex with the RAD9-RAD1-HUS1 complex (also known as 9-1-1) (276). In *S. cerevisiae,* it is believed that the 9-1-1 complex directly activates ATR under certain *in vitro* conditions, howerver it is unclear whether this activation is relevant *in vivo* (277). More convincingly, studies have shown that the 9-1-1 complex recruits the protein topoisomerase-

binding-protein-1 (TOPBP1) to ATR (278,279). TOPBP1 contains an ATR activation domain that interacts and activates ATR-ATRIP complexes *in vitro* and the overexpression of the domain by itself is sufficient for that activation (280). Interestingly, the recruitment of the 9-1-1-TOPBP1 complex and the ATR-ATRIP complex to sites of damage appear to be two independent mechanisms (281,282). This suggests a 2 hit hypothesis for the activation of the checkpoint, which might be an evolutionary signaling mechanism to prevent the inappropriate launching of the checkpoint. By having two necessary, but independent, activations, the cell is able to control more tightly the triggering of a checkpoint. The knowledge is still lacking as to whether the activating signal is the same, or whether two different signals are required for the activation of the checkpoint. As a note, multiple phosphorylation sites have been mapped on ATR, however, and unlike ATM which has an autophosphorylation site at S1987, none of the mapped sites of ATR are good predictors for its activation. As such, post-translational modifications to this date do not seem required for ATR activation. The transduction of signal from ATR to rad53 in yeast and Chk1 in mammals require claspin (283). Claspin is an adaptor molecule that is found at the replication fork and brink ATR and Chk1 together for their interaction (283). Once bound, ATR phosphorylates Chk1 on both Ser317 and Ser345, two sites which seem to be reliable indicators of Chk1 activation (284,285). Chk1 serves to transduce the signaling functions of the checkpoint from the chromatin to the whole nucleus (more on that later).

Many of the ATR crucial functions occur at the chromatin, independently form Chk1. Most of these are at the replication fork to promote its stability and the recovery of stalled replication forks. However, the exact mechanism of how ATR perform these functions is poorly understood. The substrates of ATR at the replication fork include the replication factor C complex, RPA1 and RPA2, the minichromosome maintenance complex 2-7 (MCM 2-7), MCM10 and several DNA

polymerases (286-289). MCM2 is a component of the MCM2-7 protein complex that unwind the DNA. ATR phosphorylates MCM2 on Ser108 which permits the docking of polo-like-kinase-1 (PLK1) that act on the replication fork (290). Another function of ATR is to regulate DNA repair at stalled replication forks. ATR can phosphorylate proteins that regulate recombination, and the list includes BRCA1, WRN and BLM proteins (291-293). The exact function of these ATR phosphorylations on the repair protein activities is still unclear in the literature. Finally, ATR is able to phosphorylate both FANCD2 and XPA protein1. FANCD2 regulates inter-strand crosslink repair and its phosphorylation promotes its monoubiquitinylation and localization to damage foci (294). Likewise the phosphorylation of the nucleotide-excision repair protein XPA regulates its intracellular localization (295).

As such, the initial molecular event that activates the S phase checkpoint is the coating of ssDNA by RPA which results in a temporary halt in S phase progression. The biological consequences of this ATR-Chk1-S phase checkpoint activation is to maintain the replication process intact. The mechanism of how this is achieved is unclear but it is thought that ATR is able to stabilize replication forks that contain ssDNA (296). This might occur through phosphorylation of the MCM2-7 helicase complex which prevents it from unwinding more DNA. As such, this physically stops the process so the ssDNA lesion can be repaired (297,298). It is interesting to note that the mechanisms of crosstalk between ATR and the replication machinery is largely not understood.

The intra-S phase checkpoint can also be activated by double stranded breaks. In that case, the ATM kinase is activated. ATM is normally present in the nucleus as an inactive dimer bound to protein phosphatase 2A (299). The sensor for the double stranded DNA breaks is the Mre11-Rad50-Nbs1 complex that recognizes sites of double standed breaks and recruits ATM to the site.

ATM the autophosphorylates itself which allows it to break into its monomeric form and be activated (300,301). The activation occurs within minutes of insult (such as ionizing radiation). ATM is then able to phosphorylate and activate Chk2 which results in activation of the ATM-Chk2 axis. In most cases, damaging agents are able to activate both the ATM-Chk2 and the ATR-Chk1 pathway and these pathways can have redundant as well as independent signaling functions.

There are 2 signaling effectors downstream of the ATR-Chk1 and the ATM-Chk2 pathway that appear to play major signaling role. The first is p53 which we will discuss at length below and the second is the protein phosphatase Cdc25 family. Among the latter, the most important appears to be the Cdc25a isoform in the S phase checkpoint. Upon its phosphorylation by Chk1 and Chk2, Cdc25a is eliminated by proteosomal degradation (302). This results in the accumulation of Cdk2 and stalling of the replication fork (303,304).

One of the functions of the establishment of the S phase checkpoint is to inhibit origin firing in order to prevent the generation of new origins of DNA replication while DNA is being repaired. The degradation of Cdc25a phosphatase is critical for that as it prevents the dephosphorylation and activation of the cyclinE-Cdk2 complex which prevents loading of Cdc45 replication protein onto origins (305).

The checkpoint kinases have also functions in normal regulation of origin firing. The rate of DNA cells was observed to be increased in cells lacking a functional ATM (cells derived from Ataxia Telegenctasia patients, a disease caused by mutations of ATM) (306) as well as after treatment with caffeine, a double inhibitor of ATM and ATR (307). Also, reducing Chk1 levels in cells absent treatment has been observed to cause an increase in origin firing (308,309).

2.3.3: The role of the cohesin complex

Cohesin is a complex of proteins that is involved in sister chromatid cohesion as well as numerous roles in the DNA damage pathway and the regulation of gene expression in proliferating and post-mitotic cells. The cohesion complex is formed of four core units and these include Smc1, Smc3, Scc1 (rad21) and either SA1 or SA2 (310). Two of these proteins are members of the structure maintainance of chromosome (SMC) family. The topology of the organization of this complex is thought to occur in a ring-like fashion where Scc1 acts as a linkder between Smc1 and Smc3 by binding Smc1 through its C termins and Smc3 through its N terminus. Scc1 is also associated with the fourth subunit (Scc3 in yeast and SA1 and SA2 in vertebrates) (310,311).

Sister chromatid cohesion can only be established once the DNA has been replicated during the S phase of the cell cycle. The establishment of that cohesion depends on Eco1/Ctf7, an enzyme that acts as an acetyltransferase (312,313). Its key function appears to acetylate cohesion on 2 lysine residues located on the ATPase domain of SMC3 (314). Mutating these residues causes lethality in yeast, and an Eco1 knockout can survive if yeast expresses another mutant of Smc3 that mimicks the acetylated state (314). In human cells, a related enzyme Esco1 appears to function as Eco-1 as it depletion causes acetylation defects on Smc3 (315).

Interestingly cohesion functions in controllong DNA damage. The S. pombe ortholog of Scc1, Rad21, was identified first in genetic screens for mutations that are hypersensitive to DNA damage (316). Budding yeast experiments suggested that cohesion mutants are defective in repairing damage DNA (317) and that this is dependent on the ability of cohesion to mediate sister chromatid cohesion, presumabely because DNA double stranded breaks are repaired by recombination between sister chromatids, an event facilitated by the cohesion complex.

Most of the studies on how cohesion is regulated by DNA damage were done in budding yeast. Following DNA damage, cohesion accumulates around the site of the damage independent of whether the break occurred in a cohesion binding region or not. This process is dependent on the proteins Scc2 and Scc4 (318,319). It is interesting to note that DNA damage can also allow the establishment of sister chromatid cohesion outside of S phase and in G2 phase. This event depends, much like in S phase, on Eco1 (314,320). Recent evident suggest that cohesion establishment following DNA damage depends on Mec1 (yeast ortholog of ATR) and Chk1 which appear to phosphorylate Scc1 on serine 83 (321). These observations point to Scc1 as the key target of the DNA damage response with its respect to its ability to initiate cohesion. Much less studies have been done in vertebrates on cohesion but the general consensus is that, similar to yeast, cohesion is also recruited to sites of DNA breaks in a process that depends on the Smc5/Smc6 complex (322). The exact regulation at these sites is not well understood in vertebrates however. As in budding yeast, cohesion is phosphorylated following DNA damage but this phosphorylation occurs on Smc1 and Smc3 and is mediated by both ATM and ATR (323-325). In fact, nonphosphorylatabel mutant of Smc1 reduces DNA repaire efficiency in irradiated cells much like Ataxia Telegenctasia cells indicating that Smc1 might be one of the key targets of ATM in the DNA damage response (323,324,326). Interestingly these cells also continue to synthesize DNA after damage suggesting that the cohesion complex might be essential for the activation of the S-phase checkpoint. After DNA damage (317). However, it remains to be clarified how this contributes to the Sphase checkpoint and whether other molecules, part of the cohesion complex, are involved in the S phase checkpoint. A summary of the S phase checkpoint and its regulation is provided in figure 4.

2.4 Targeting the DNA damage pathway in cancer therapeutics

Most of the research conducted about the DNA damage pathway aims to delineate its biochemical regulation, understand its signaling function, and assess its potential use in cancer therapy. On the latter subject, the data is conflicting. However, recently, there is increased appreciation of the potential benefits of targeting DNA damage pathways, especially in conjunction with DNA damaging chemotherapeutics. The idea is very simple: target the pathway that constitutes the cancer cell defense mechanism against these chemotherapeutics.

None illustrates this concept better than the tumor suppressor p53. Traditionally, mutations in p53 have been associated with higher malignancy and increased proliferative, invasive and metastatic potential (327,328). However, a change in perspective regarding p53 began emerging in the context of chemotherapy. Studies have shown that mice models of genetic breast cancer harboring a functionally null p53 respond better to doxorubicin chemotherapy than mice harboring the wild type allele (329). This has been attributed to the ability of wild type tumors to undergo both cell cycle arrest and senescence (329). In comparison, the mutant p53 failed to arrest or undergo senescence which resulted in aberrant mitosis and eventually cell death and improved outcomes in mice. However, since targeting p53 in synergy with chemotherapy is not viable option due to its tumor suppressive functions, research have focused more recently on the potential of targeting the kinases involved in the DNA damage such as the Chk kinases.

Of the checkpoint kinases, Chk1 targeting garnered the most interest in the literature. Chk1 activity is regulated by its activation (discussed previously) and its degradation. As a matter of fact, Chk1 degradation has been observed in response to many agents (330,331). This is due to its ubiquitination and its proteosomal degradation by the Skp1-Cul1-Fbx6 and/or Cul4A-DDB1 ubiquitin E3 ligases (331-333). Importantly the cell cannot tolerate reduction in Chk1 activity and this leads to spontaneous cell death (334). This is most probably because of the inability of the cell

to maintain the replication fork and hold at S or G2 phase in the presence of replication stress. This may be one of the mechanisms by which many of the anticancer drugs exercise their tumor suppressive functions. These observations led to the idea that in cancers that have defects in downregulation of Chk1, these chemotherapeutics would be less efficient.

Chk1 protein levels following the use of DNA damaging agents is directly correlated with the tumor chemosensitivity. For example, cells that elicited extraordinary resistance to camptothecan did not degrade Chk1 following the treatment (335). Depletion of Chk1 by siRNA dramatically sensitized those cells. These data may point to a general mechanism by which tumors acquire resistance to chemotherapy, which ultimately facilitate the survival of the resistant clones. As such, loss of function of Chk1 is very rarely found in tumors (336) and most of the changes include robust overexpressions in tumors in comparison to adjacent normal tissue, as well as positive correlation of Chk1 expression with tumor stage and grade (337-340). Interestingly, another rationale for targeting Chk1 is emerging in tumors with mutations in p53. These mutant tumors have lost the ability to arrest in G1 phase die to the loss of p53 and as such are much more reliant on both S and G2 phases and recent work using a humanized mouse model of triple negative breast cancer confirmed these ideas (341,342). As such, numerous attempts were made by pharmaceutical companies to identify specific Chk1 inhibitors in order to use in synergy with chemotherapy. However, a major issue has been the off-targets effects and toxicity associated with these Chk1 inhibitors, the chemotherapy or their combination. The major toxicity has been reported to be hematological. Therefore, there is a need for alternative strategies to be developed to target Chk1.

Recent work has demonstrated that mutations of Chk1 at G448 and L449 disrupt its closed conformation and renders it constitutively active (343). The expression of this form of Chk1

completely blocked cancer cell proliferation and led to cell death under normal growth conditions (343). This unusual phenotype is probably due to the fact that the mutant raises a “false” alarm as if the cell has a lot of damage beyond its ability to repair and as such activates cell death mechanisms. This has led to the concept that too much activation of Chk1 is detrimental to cell survival in the absence of DNA damage. As such, a potential therapeutic strategy could be the artificial activation of Chk1 in absence of DNA damage. The advantage of this strategy is that it precludes the use of toxic chemotherapeutic drugs and therefore reduces a lot of the side effects of cancer treatments.

2.5 Conclusion

The understanding of the processes that regulate cell cycle control have improved over the past two decades. However, the studies of the S phase checkpoint has lagged behind as its importance was underestimated initially. As the molecular mechanisms of that checkpoint unravel, exciting signaling discoveries are being made that could impact the future of cancer therapy

Chapter 3

P53-dependent upregulation of neutral sphingomyelinase-2;

Role in doxorubicin-induced growth arrest

p53-dependent upregulation of neutral sphingomyelinase-2; Role in doxorubicin-induced growth arrest

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Running title: nSMase2 is required for doxorubicin growth arrest

ABSTRACT

Neutral sphingomyelinase-2 (nSMase2) is a ceramide-generating enzyme that has been implicated in growth arrest, apoptosis, and exosome secretion. While previous studies have reported transcriptional upregulation of nSMase2 in response to daunorubicin, through Sp1 and Sp3 transcription factors, the role of the DNA damage pathway in regulating nSMase2 remains unclear. In this study we show that doxorubicin induces a dose-dependent induction of nSMase2 mRNA and protein with concomitant increases in neutral sphingomyelinase activity and ceramide levels. Upregulation of nSMase2 was dependent on ATR, Chk1 and p53, thus placing it downstream of the DNA damage pathway. Moreover, overexpression of p53 was sufficient to transcriptionally induce nSMase2, without the need for DNA damage. DNA-binding mutants as well as acetylation mutants of p53 were unable to induce nSMase2 suggesting a role of nSMase2 in growth arrest. Moreover, knockdown of nSMase2 prevented doxorubicin-induced growth arrest. Finally, p53-induced nSMase2 upregulation appears to occur via a novel transcription start site upstream of exon 3. These results identify nSMase2 as a novel p53 target gene, regulated by the DNA damage pathway to induce cell growth arrest.

3.1 Introduction:

Ceramide is a bioactive sphingolipid that has been implicated in numerous biological processes, such as cell cycle arrest and cell death (2). Ceramide can be generated through *de novo* synthesis, salvage of sphingosine, or hydrolysis of sphingomyelin (344). The latter reaction involves the action of sphingomyelinases, a class of enzymes that differ in their cellular localization and pH optima for activity (82,84). Neutral sphingomyelinase-2 (nSMase2) is the most studied of the neutral sphingomyelinases, and nSMase2 activation has been implicated in growth arrest, apoptosis, and exosome secretion (345).

Activation of nSMase2 occurs through different mechanisms. *In vitro* nSMase2 activity is regulated by anionic phospholipids via binding to positively charged sites on the N-terminus of the protein (85,96,98). Post-translational activation occurs also via phosphorylation on five serine residues in response to oxidative stress through a p38 mitogen-activated protein kinase mechanism (99,346). Recently, transcriptional upregulation of nSMase2 became appreciated as a mechanism of enzyme activation. As such, upregulation of nSMase2 was shown to be mediated by Sp1 and Sp3 through direct binding to the nSMase2 promoter in response to All trans retinoic acid (ATRA) and daunorubicin (103,104). Moreover, Runx2 was shown to regulate nSMase2 transcriptionally in response to bone morphogenic protein-2 (347).

Chemotherapeutics are potent generators of ceramide (348). The anthracyclin doxorubicin, a daunorubicin analogue, is used as a first line chemotherapeutic agent for the adjuvant treatment of many tumors such as breast and lung neoplasms (349,350). Multiple mechanisms of action of doxorubicin have been elucidated, but its major anti-tumor activity is thought to occur through the generation of DNA breaks in the tumor cell. Specifically, doxorubicin binds topoisomerases and

stabilizes their interaction with DNA, which prevents the rejoining of nicked DNA following relaxation of supercoils to create DNA breaks (351).

Following DNA breaks, the DNA damage response is the canonical pathway activated in cells and serves as a protective mechanism to halt cell growth and repair damage (352,353). Mechanistically, the activation is thought to occur in response to both single stranded breaks as well as double strand breaks. The different stimuli lead to the activation of different effector kinases (ATR and Chk1 for ssDNA breaks, ATM and CHk2 for dsDNA breaks) (354,355). The signaling cascade converges on p53, which acts as a central hub in integrating signals and regulating effector biologies (356). Mutations of p53 occur most commonly in its DNA-binding site and affect its transcriptional activity. As such, these mutations are associated with tumorigenesis, as well as worse outcomes of existing neoplasms (357).

Ceramide generation in response to anthracyclines has been implicated in mediating anti-tumor effects such as cell death and growth arrest (41,358,359). However, the precise mechanism of generation of ceramide as well as its regulation, remain ambiguous. Moreover, ceramide generation in response to p53 activation has been studied, yet contradicting reports have emerged. While it has been reported that ceramide is an upstream regulator of p53 (54,360,361), other evidence suggests that p53 regulates ceramide generation in response to specific stresses (362-364). Understanding the regulation of ceramide generation would provide better understanding of tumor responsiveness to doxorubicin. Recently, the effect of daunorubicin on nSMase2 has been described. Daunorubicin activates nSMase2, concomitant with an increase in ceramide formation (103).

In this study, we first investigate whether doxorubicin, a daunorubicin analogue that is clinically relevant in breast cancer therapy, leads to nSMase2 upregulation and increased ceramide generation in MCF7 breast cancer cells and uncovers the specific mechanism of nSMase2 transcriptional regulation in response to doxorubicin. The data demonstrate that nSMase2 is a downstream target of p53 and is more selectively regulated by ATR and Chk1. Notably, nSMase2 transcriptional activation occurs via a novel transcription start site (TSS). Taken all together, these studies identify nSMase2 as an important component of the DNA damage pathway.

3.2 Materials and Methods

3.2.1 Materials

MCF7 breast carcinoma cells were obtained from ATCC (Manassas, VA). RPMI culture medium, fetal bovine serum and SuperScript reverse transcriptase were obtained from Invitrogen. Antibodies for nSMase2 (H195), Sp1 (PEP2), Sp3 (D20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Chk1 (2360), Chk2 (2662), ATM (2873), ATR (2790), p53 (9282) and Actin (4967) were from Cell Signaling Technologies (Beverly, MA). The inhibitors AZD7762 (S1532) MK-8776 (S2735) were from Selleck Chem (Boston, MA). Go6976 (2253) was purchased from Tocris Biosciences (Bristol, UK). Bisindolylmaleimide I (13298) was obtained from Cayman Chemicals (Ann Arbor, MI). siRNA for TP53 (s607) and CREB3L1 (s40546) were from Life Technologies (Grand Island, NY). All other siRNAs, CHEK1 (SI00024570) CHEK2 (SI00095305) ATM (SI00000840) ATR (SI00023107) Sp1 (SI150983) Sp3 (SI0004788) were from Qiagen (Hiden, Germany). Porcine brain sphingomyelin and phosphatidylserine were from Avanti Polar Lipids (Alabaster, AL). Doxorubicin, actinomycin D, and, unless indicated otherwise, all other chemicals were obtained from Sigma (St Louis, MO)

3.2.2 Cell culture and siRNA

MCF-7 cells were grown at 37 °C, 5% CO2 in 10% fetal bovine serum in RPMI. Cells were subcultured in 60-mm dishes (175, 000 cells) and medium was changed 1 hour before the start of experiments. For siRNA experiments, cells were plated in 60-mm dishes (100, 000 cells) and 24 hours later transfected with 10 nM negative control or 10 nM of the desired siRNA using Lipofectamine RNAiMAx reagent (Life Technologies) according to manufacturer's protocol. After 24 hours, cells were incubated in fresh medium for 1 hour prior to treatment.

3.2.3 Construction of p53 mutants and cellular overexpression

The plasmid pcDNA-WTp53 was a generous gift from Dr. Ute Moll. The R280K, K120R, and K161R mutants were produced using quick change site-directed mutagenesis (Agilent technologies). The primers used for the mutants are:

R280K	F	TGTGCCTGTCCTGGGAAAGACCGGCGCACAGAG
	R	CTCTGTGCGCCGGTCTTTCCCAGGACAGGCACA
K120R	F	GCATTCTGGGACAGCCAGGTCTGTGACTTGCACGTACTCCCCTGC
	R	GCAGGGGAGTACGTGCAAGTCACAGACCTGGCTGTCCCAGAATGC
K161R	F	GCACGTACTCCCCTGCCCTCAACAGGATGTTTTGCCAACTGGC
	R	GCCAGTTGGCAAAACATCCTGTTGAGGGCAGGGGAGTACGTGC

For overexpression of vectors in mammalian cells, MCF-7 cells were subcultured in 60 mm dishes (350, 000 cells) and medium was changed 1 hour before the experiment and then were transfected with 1μM of the plasmid containing the insert of interest or its complementary empty vector using Xtreme gene transfection reagent (Roche) according to the manufacture's protocol. Cells were collected 24 hours later and further experiments conducted.

3.2.4 Protein extraction and immunoblotting

For cellular protein extraction, cells were scraped in 0.75% SDS and lysed by sonication. Bradford reagent (Bio-Rad) was used to determine protein concentration prior to immunoblotting. Lysates were mixed with equal volumes of 2x Laemelli buffer (Bio-Rad) and boiled for 5 minutes. The protein was separated by SDS-PAGE using the Criterion gels (Bio-Rad) and immunoblotted as described previously (63).

3.2.5 Quantitative Real Time PCR

RNA extraction was performed using the Purelink RNA kit (Life Technologies) according to manufacturer's protocol. RNA quality and concentration was verified by nanodrop after which 1 μg of RNA was transformed into cDNA using the Quanta cdna kit according to manufacturer's protocol. For qRT PCR, reactions were run in triplicates in 96 well plates with each reaction containing 10 uL of 2X iTAQ mastermix, 5 uL of cdna, 1 uL of taqman primer-probe and 4 uL of water. The primer-probes used were purchased from life technologies and amplified nSMase2 (Cat# 4331182), Actin, (Cat# 4448484) and CREB3L1 (Cat# 4331182).

3.2.6 Neutral Sphingomyelinase Assay

Neutral sphingomyelinase activity was assayed as described previously using ^{14}C-[methyl]sphingomyelin as substrate (63). Briefly, SM from bovine brain (Avanti polar lipids) and C14 PS (Avanti polar lipids) were dried under N2(g) and resuspended in Triton X-100 mixed micelles by sonication. The final reaction conditions contained 0.1% Triton X-100, 100 mM Tris buffer, pH 7.5, 20 mM $MgCl_2$, and 5 mM DTT with 5 mol% SM (SM only) or 5 mol% SM + 5 mol% PS (SM + PS). Cellular lysates containing 80 μg of protein were added to 100 μL of assay buffer and were incubated for an hour. After that, reaction was quenched and a modified Bligh and Dyer extraction was performed. The aqueous phase (700 μL of supernatant) was transferred to scintillation vials containing 3 mL of scintillation fluid and counted.

3.2.7 Analysis of Cellular Sphingolipids

Prior to collection, cells were incubated in serum-free medium (RPMI with 0.1% fatty-acid free BSA) for 3 hours. Cells were scraped and pelleted and extraction and analysis by LC/MS mass spectrometry was performed as described previously (63). Lipids were normalized to total phosphate levels of selected sample.

3.2.8 Promoter cloning and luciferase assays

Using human genomic DNA (Roche), we amplified 1500 bp upstream of exon1 using the following primers 5'-CGGCTCGAGGGAGGTGTATGTGAATGAGGTTCC-3' and 5'-CCCAAGCTTGGGTCCGGAGCCTCCCTCAGACTC-3'. The insert was purified and cloned into a pGL3 basic vector (life technologies). Cells were transfected with equal plasmid amount of pGL3 containing insert and a control plasmid containing Beta-galactosidase. After 24 hours, cells

were treated with Doxorubicin and then assayed for promoter activation using a luciferase assay kit. Results were normalized to B-galactosidase activity as measured by a beta-galactosidase assay (Agilent)

3.2.9 Statistical analysis

All experiments are n of 3 unless expressed otherwise. Bars are representative of means and standard deviation. Two-way ANOVA was employed for the analysis of samples with multiple variables (for example treatment and siRNA) and one-way ANOVA was employed for the rest. Significance is achieved at $p<0.05$

3.3 Results

3.3.1 nSMase2 is induced by doxorubicin in MCF7 cells

Upregulation of nSMase2 was reported in response to daunorubicin, in MCF7 breast cancer cells (103). However, since daunorubicin is not used in breast cancer therapy, we sought to study NSMase regulation and ceramide generation using doxorubicin as a clinically relevant chemotherapeutic drug in breast cancer. Doxorubicin treatment of MCF7 cells increased nSMase2 protein levels in a dose (Fig. 5A-B) and time-dependent manner (data not shown), with maximal nSMase2 induction occurring with 600 nM doxorubicin at 24 hours. This was concomitant with an increase in total *in vitro* neutral sphingomyelinase activity (Fig. 5C) and total cellular ceramide levels (Fig. 5D). Analysis of ceramide according to chain length revealed that C14 and C16 are the major ceramide species upregulated in response to doxorubicin treatment (Fig. S1A), while sphingosine (Fig. S1B) and sphingosine-1-phosphate (Fig. S1C) levels also increased. To evaluate

the upregulation of other neutral sphingomyelinases, mRNA levels of all 3 cloned human neutral sphingomyelinases were analyzed by quantitative real-time PCR. This revealed that nSMase2 was the only N-SMase enzyme induced transcriptionally and was upregulated by around 60 fold (Fig. 5F). Importantly, knockdown of nSMase2 by siRNA abolished the doxorubicin-mediated increase in total NSMase activity (Fig. 5E). Thus, taken together, these results demonstrate specific induction of nSMase2 by doxorubicin in conjunction with an increase in ceramide levels.

3.3.2 nSMase2 is upregulated transcriptionally via a novel transcription start site (TSS)

Since the levels of nSMase2 mRNA increased, it was prudent to determine if the effect was purely transcriptional. To rule out effects of doxorubicin on mRNA stability, cells were treated with doxorubicin for 24 hours, after which actinomycin D was added. As seen in Fig. 6A, nSMase2 mRNA stability was similar in both vehicle-treated and doxorubicin-treated cells. To determine if the effect is through promoter activation of nSMase2, the putative promoter of nSMase2, encompassing the first 1000 base pairs upstream of exon 1, was cloned, and its activity was evaluated by luciferase reporter assays. Doxorubicin-treated MCF7 cells displayed a 2.5 fold increase in luciferase activity that suggested activation of the putative promoter (Fig. 6B). However, since the fold change in promoter activity and mRNA levels did not match (2.5 fold versus 60 fold), it was important to investigate the presence of an alternative promoter. The annotated nSMase2 5'-untranslated region (UTR) encompasses exons 1 and 2 as well as part of exon 3. Using intronic-exonic primers, heteronuclear RNA (unspliced mRNA) of nSMase2 was amplified. The results suggested that the 5'-UTR of nSMase2 mRNA, upregulated after doxorubicin treatment, does not include exon 1 and exon 2 as exon1-intron2 primers failed to amplify to the same extent as downstream exonic-intronic junctions (Fig. 6C)..These results point

to a doxorubicin-specific transcriptional regulation of nSMase2 through a novel transcription start site upstream of exon 3, but not including exon 1 and 2.

3.3.3 nSMase2 transcriptional activation is independent of known transcriptional regulators

Transcriptional regulation of nSMase2 has been described in response to different stimuli. Sp1 and Sp3 were shown to regulate nSMase2 transcriptionally in response to daunorubicin and ATRA (103,104). Formation of reactive oxygen species (ROS) was shown to modulate nSMase2, and doxorubicin is a potent generator of ROS (141,142,364). In addition, Denard et al. described regulation of ceramide production by CREB3L1 following doxorubicin in MCF7 cells (365). As such, it was next essential to determine the effect of these transcriptional regulators on nSMase2 in response to doxorubicin. As can be seen, siRNA knockdown of Sp1 and Sp3 did not prevent nSMase2 upregulation after doxorubicin treatment (Fig. 7A). Moreover, pre-treatment with N-acetylcysteine, a quencher of ROS, had no effect of nSMase2 induction (Fig. 7B). Finally, knockdown of CREB3L1 by siRNA did not inhibit nSMase2 upregulation in response to doxorubicin (Fig. 7C). CREB3L1 knockdown was verified by qRT-PCR (Fig. S2). Taken together, these results suggest that nSMase2 transcriptional activation is independent of known regulators, and is possibly due to a new previously undescribed mechanism.

3.3.4 nSMase2 is regulated transcriptionally by specific components of the DNA damage pathway

Ito et al. described an effect of Bis (a classical and novel PKC inhibitor) on nSMase2 in response to ATRA (104). In our study pre-treatment with Go6976 (a classical PKC inhibitor) exerted a robust inhibitory effect on nSMase2 induction, while Bis pre-treatment had a mild effect (Fig. 8A-B). These results were difficult to reconcile since Bis should also inhibit the classical

PKCs and suggested an off-target effect of Go6976. Typically, Go6976 is used as a PKC inhibitor at concentrations that range between 1 and 3 μM. To evaluate the optimal concentration for the effects of Go6976, cells were pre-treated with different concentrations of Go6976 and nSMase2 induction was evaluated. Unexpectedly, inhibition of nSMase2 induction occurred at concentrations as low as 300 nM at the protein level (Fig. 8C) and 100 nM at the mRNA level (Fig. 8D). These results suggested that PKCs are not involved in mediating nSMase2 induction.

The best characterized off-target of Go6976 is the checkpoint kinase Chk1 which is inhibited by Go6976 at nM concentrations (366). Moreover, doxorubicin is a DNA damage agent that activates effector kinases of the DNA damage response (367). Therefore, it was rational to hypothesize that nSMase2 transcription may be regulated by Chk1 and other DNA damage regulators. Using the nonspecific Chk1/Chk2 inhibitor AZD7762, as well as the specific Chk1 inhibitor MK-8776, a robust downregulation of nSMase2 induction was observed both at the protein (Fig. 9A) and mRNA level (Fig. 9B). Individual knockdown of Chk1 and Chk2 revealed that Chk1 is the major isoform responsible for nSMase2 induction in response to doxorubicin at the protein level (Fig. 9C), as well as the mRNA level (Fig. 9D).

Since Chk1 is an effector kinase of the DNA damage response that is in turn activated by upstream kinases, namely ATM and ATR, we undertook determining the signaling pathway upstream of Chk1 that controls nSMase2 induction. To determine whether ATM or ATR influence nSMase2 upregulation, individual siRNA knockdown of ATM and ATR was performed. ATR knockdown significantly downregulated nSMase2 induction both at the protein (Fig. 10A) and mRNA level (Fig. 10B). In contrast, knockdown of ATM had very modest effects (Fig. 10A). These results place nSMase2 as a downstream transcriptional target of both ATR and Chk1.

3.3.5 p53 is both necessary and sufficient for nSMase2 transcriptional upregulation

The main transcriptional functions of ATR and Chk1 occur through the activation of the tumor suppressor p53. Therefore, it became important to determine whether p53 was required for nSMase2 induction in response to doxorubicin. Knockdown of p53 by siRNA prevented nSMase2 induction in response to doxorubicin both at the protein (Fig. 11A) and the mRNA levels (Fig. 11B). Reciprocally, overexpression of p53 yielded a dose dependent increase in nSMase2 expression in the absence of doxorubicin (Fig. 11C). To confirm that this is dependent on the transcriptional activity of p53, a p53 construct carrying the common mutation R280K in the DNA binding domain was used to test for nSMase2 induction upon its overexpression. Overexpression of WT p53 but not mutant R280K p53 resulted in nSMase2 induction in the absence of genotoxic stress (Fig 11D). To validate these results, MDA-MB-231 breast cancer cells, that carry the p53-R280K mutation, were stimulated with doxorubicin. As can be seen, doxorubicin treatment had no effect on nSMase2 mRNA levels (Fig. 11E) in these cells. Furthermore, activity assays did not show significant increase in NSMase activity (Fig. 11F). Collectively, these results demonstrate that p53 is both necessary for nSMase2 transcriptional activation in response to doxorubicin and sufficient to upregulate nSMase2 transcriptionally in the absence of genotoxic stress.

3.2.6 nSMase2 mediates growth arrest in response to doxorubicin

Lysine mutants of p53 have become the subject of increasing interest. Studies have shown that these mutants do not affect the tumor suppressive functions of p53 in murine models, yet they impair p53-mediated growth arrest and apoptotic functions. Therefore, the ability of these mutants to induce nSMase2 was evaluated through p53 mutant constructs harboring lysine to arginine mutations at positions 120, 161, and both 120 and 161. While the K120R mutant induced nSMase2,

the K161R as well as the K120-161R double mutants did not in comparison to WT p53 (Fig. 12A). The specific nature of nSMase2 induction in response to WT p53 but not the K161R mutant suggested possible biological functions of nSMase2 upregulation in response to doxorubicin. As such, the involvement of nSMase2 in mediating either apoptotic or growth arrest functions in response to Doxorubicin was studied. At the concentrations at which it induces nSMase2, doxorubicin did not induce cell death and nSMase2 knockdown did not change trypan blue uptake (Fig. 12B). On the other hand, BrdU analysis revealed that, while doxorubicin-treated control cells did not incorporate BrdU, the cells with nSMase2 downregulation had increased uptake of BrDU (Fig. 12C). Taken together, these results suggest a role of nSMase2 in mediating growth arrest following doxorubicin treatment.

3.4 Discussion

In this study, we have explored the regulation of nSMase2 and ceramide generation in breast cancer cells treated with Doxorobucin. We report that nSMase2 is the primary N-SMase regulated by Doxorubicin and that this is independent of reported transcriptional regulators of nSMase2. Instead, nSMase2 induction was strongly dependent on p53, ATR and Chk1, and potentially occurs through an alternate transcriptional start site in the nSMase2 gene. Collectively, these data define a novel pathway of nSMase2 regulation by DNA damage effector proteins, and the results shed light on mechanisms of ceramide generation by Doxorubicin in breast cancer.

A number of studies have implicated N-SMase activity in the cellular responses to cytokines and stress including chemotherapeutics (202,214,359,368-370). However, little is known about the specific N-SMase isoforms involved, nor the mechanisms of their regulation. In the current study, nSMase2 was identified as the major doxorubicin responsive N-SMase in breast

cancer cells. Previously, nSMase3 was shown to be upregulated following doxorubicin treatment acutely to mediate cancer cell sensitivity to the drug (371). However, recent data raised doubt on whether nSMase3 functions as a sphingomyelinase (372).

Notably, nSMase2 induction was transcriptional, as evidenced by effects on hnRNA and lack of effects on mRNA stability, and consistent with a previous study using the doxorubicin analogue, daunorubicin. However, this induction appears to be through a novel regulatory pathway involving the tumor suppressor protein p53, and the DNA damage effectors ATR and Chk1 – as evidenced by both pharmacological inhibitors and siRNA knockdown. Furthermore, p53 overexpression was sufficient to induce nSMase2 transcriptionally, even in the absence of genotoxic stress. These results differ from daunorubicin induction of nSMase2 in MCF7 cells, which was reported to require the transcription factors Sp1 and Sp3 – which appear to be dispensable in our system (103). Notably, the previous study relied on pharmacological inhibitors and thus off-target effects could account for the differences observed. The lack of role of Sp1 and Sp3 in the doxorubicin response is also consistent with observations suggesting an alternative transcription start site for nSMase2. In the previous study, Sp1 and Sp3 were observed to interact with the nSMase2 gene upstream of exon 1. In contrast, doxorubicin does not significantly increase transcription through exon 1 or exon 2 of the Smpd3 gene as evidenced by both RNA sequence and hnRNA analysis with specific exon-intron primers. While attempts to fully define this alternate TSS have thus far been unsuccessful, it should be noted that multiple TSS sites are highly common for genes with long first introns as is seen with the Smpd3 gene (>70kB).

Importantly, the linking of nSMase2 expression to both ATR and Chk1 suggests that nSMase2 induction by p53 may occur in very specific cellular contexts, rather than functioning as a general effector of DNA damage. Indeed, ATR and Chk1 activation is known to depend on

ssDNA breaks generated following genotoxic stress (355) and doxorubicin (as well as daunorubicin) is able to induce both ssDNA and dsDNA breaks. Notably, UV radiation – another activator of ssRNA breaks, ATR and Chk1 - has also been described to induce N-SMase activity, and tumor resistance to apoptosis by UV has been linked to failure of N-SMase activation (373,374). Although the specific NSMase as well as its regulation had not been studied; our study suggests that nSMase2 could be the isoform also responsible for NSMase activity increase in response to UV. These data would predict that stimuli that activate the ATM-Chk2 axis such as gamma irradiation (IR) would not induce nSMase2. Indeed, although IR has been reported to generate ceramide, this was attributed to the activation of acid sphingomyelinase and not N-SMase and was also reported to be p53-independent (375,376). Further to this, a number of studies have reported p53-dependent generation of ceramide in response to different stresses; however, there is a paucity of information on the underlying mechanisms by which p53 performs these functions (371,377). To date, only two studies have focused on regulation of specific sphingolipid enzymes by p53. The first showed loss of sphingosine kinase 1 following genotoxic stress occurred in a p53 dependent manner (378), and the second elucidated a mechanism of p53-dependent induction of CerS6 in response to folate stress (362). However, this clearly suggests that the pathway of ceramide generation is dependent on the specific stress, the cellular context and, as noted above, activation of additional signaling pathways. Furthermore, the upregulation of wild-type p53 in response to chemotherapy can mediate cancer cell resistance to these agents through activation of pathways of cell cycle arrest and DNA repair. Since ceramide has been proposed to regulate growth arrest in response to p53, a wider understanding of the differential regulation and effects of specific ceramide-generating pathways is of paramount importance. This would allow the targeting of specific enzymes through inhibitors in conjunction with chemotherapy to promote its efficacy.

Finally, the identification of genes that are differentially regulated by wild type versus mutant p53 is gaining increasing importance with the study of both loss and gain of functions of mutant p53 attempting to identify reasons behind the aggressiveness of cancers with mutations in p53. Given the established roles of ceramide generation in tumor suppressive biologies such as growth arrest, senescence, and apoptosis, as well as anti-invasiveness, one possible hypothesis for the aggressiveness of p53 mutant tumors is its inability to activate or degrade ceramide-metabolizing enzymes. The lack of effect of such p53 mutants on nSMase2 induction would be consistent with such a hypothesis. Consequently, manipulation of the sphingolipid pool in cancer cells by targeting the sphingolipid enzymes might offer a viable therapeutic approach that needs further exploration and clarification.

In conclusion, this study identifies nSMase2 as the major neutral N-SMase upregulated in response to Doxorubicin, a first-line agent used in the treatment of breast cancer, and suggests that nSMase2 is a primary pathway of ceramide generation in the doxorubicin response. Moreover, nSMase2 induction is dependent on ATR, Chk1 and p53. Collectively, these results place nSMase2 as an essential part of the DNA damage pathway. Further investigation of the potential roles of nSMase2 in this pathway could provide better understanding of the biological relevance of its activation as well as potential benefits of targeting nSMase2 in conjunction with chemotherapy.

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Appendix A

Distinct sphingolipid profiles activated by different doses of doxorubicin

A1. Introduction:

Doxorubicin is a topoisomerase poison used as a chemotherapeutic in the treatment of several human malignancies, including breast cancer. Its primary mechanism of action is through the generation of ssDNA and dsDNA breaks which result in genomic damage that leads to cell cycle arrest and, if the damage is unrepairable, apoptosis. The effector mechanisms of these biologies are the subject of intense scrutiny as many signaling enzymes and molecules are yet to be identified. The complete characterization of these effector mechanisms could have a beneficial effect on improving the efficacy of chemotherapy and sensitizing resistant cells to doxorubicin.

Sphingolipids are a class of bioactive lipids that have been implicated in many biologies. Their levels have been shown to increase following doxorubicin treatment in a recent metabolonomic profiling of MCF-7 (379). However, the mechanism of these sphingolipid changes was not assessed and their study could provide insight on how to modulate sphingolipid levels following doxorubicin treatment to favor a certain biology. The study of sphingolipid enzymatic changes following DNA damage in general, and doxorubicin in particular, is not very developed in the literature. Some of the changes were described. As a matter of fact, SK1 was shown to be downregulated by doxorubicin in a p53-dependent manner in MOLT-4 (378). Moreover, glucosylceramide synthases (GCS) is induced transcriptionally following doxorubicin in an Sp1-dependent manner (380). Further studies implicated GCS in doxorubicin resistance and the expression of P-glycoprotein and MDR-1 (381,382). Finally, a recent study showed than in Huh7 cells, doxorubicin activated *de novo* synthesis of ceramide which results in translocation of the transcription factor CREB3L1 to the nucleus (365).

A2. Materials and methods:

A2.1 Materials and cell culture

MCF7 breast carcinoma cells were obtained from ATCC (Manassas, VA). RPMI culture medium, fetal bovine serum and SuperScript reverse transcriptase were obtained from Invitrogen. MCF-7 cells were grown at 37 °C, 5% CO2 in 10% fetal bovine serum in RPMI. Cells were subcultured in 60-mm dishes (175, 000 cells) and medium was changed 1 hour before the start of experiments. Antibodies for nSMase2 (H195). qRT PCR custom arrays were purchased from Qiagen. Doxorubicin was purchased from sigma and TP53 plasmid was a generous gift from Dr. Ute Moll.

A2.2 MTT assays

Cells were seeded in 6 well plates. Following the end of the experiment, media was aspirated and cells were incubated in 1 mL of media and 1 mL of MTT reagent at a concentration of 5 mg/mL for 30 minutes. Media was then aspirated and 2 mL of DMSO were added for 5 minutes after which 200 μL aliquots were taken in duplicates and absorbance was measured at 570 nm.

A2.3 Cell cycle analysis by flow cytometry

Cells were collected and washed 2x with PBS then fixed with 1 mL of 70% ethanol overnight. The next day, cells were pelleted, ethanol removed and washed twice with PBS and incubated with 0.5 mL of PI/RNase solution (cell signaling) supplemented with 0.2% Triton X-100 for 1 hour. Cells were analyzed on a FACSCalibur machine and data were analayzed using the ModFit software

A2.4 RNA sequencing and Real Time PCR

RNA extraction was performed using the Purelink RNA kit (Life Technologies) according to manufacturer's protocol. RNA quality and concentration was verified by nanodrop. The RNA was either sent to the New York genome center for analysis by RNA sequencing or 1 μg of RNA was transformed into cDNA using the Quanta cdna kit according to manufacturer's protocol. For qRT PCR arrays, reactions were run using SYBR green. Each plate was custom ordered from Qiagen and the design of the primers can be found in supplementary figure 3.

A2.5 Analysis of Cellular Sphingolipids

Prior to collection, cells were incubated in serum-free medium (RPMI with 0.1% fatty-acid free BSA) for 3 hours. Cells were scraped and pelleted and extraction and analysis by LC/MS mass spectrometry was performed as described previously (63). Lipids were normalized to total phosphate levels of selected sample.

A3. Results:

A3.1 Doxorubicin induces distinct biologies at different doses

Doxorubicin is a potent chemotherapeutic used as a first-line treatment as a single agent or in combination therapy for the treatment of primary breast cancer. Most of the studies on doxorubicin use it as a single agent at a single dose to study specific functions normally related to DNA damage. However, there is little appreciation of the diverse biologies that doxorubicin induces at different doses. To further study this, a dose response was performed to look at cellular morphology on MCF-7 cells. The results showed that frank cell death started appearing at the 800 nM dose and above. This was confirmed by MTT assays as the higher doxorubicin doses showed decreased MTT readings, while the lower doses showed slowed growth (Fig. 13A). To check whether these low doses of doxorubicin were inducing cell cycle arrests, we looked at the cell

cycle profile of the dose response by flow cytometry. Interestingly, at 200 nM doxorubicin appears to induce a G2/M arrest consistent with what it reported in the literature. However, as the dose go up (400-800 nM), there is an S phase population that is increased in addition to the G2/M population. This suggests that at these doses of damage, the cell activates both an S and a G2/M checkpoint (Fig 13B). Finally, using RNA sequencing technology, we looked at the pathways that were selectively upregulated by doxorubicin at 600 nM as well as the pathways that were selectively downregulated. As expected, many of the pathways activated were involved in the DNA damage response such as the MAPK signaling pathway, the p53 signaling pathway as well as apoptotic pathways. However, two very interesting, previously unreported pathways, were activated. The first is that of cell adhesion molecules and adherent junctions (more on that in Appendix B), and the second is that of sphingolipid metabolism (Fig 13C). The downregulated pathways on the other hand encompassed DNA repair, cell cycle progression as well as metabolic pathways. Taken together, these results suggest that the effect of doxorubicin is dose-dependent and sheds the light on novel biological pathways regulated by doxorubicin that might contribute to its anti-tumor functions.

A3.2 Doxorubicin regulates sphingolipid metabolizing enzymes in a dose-dependent manner

From the RNA sequencing, many of the sphingolipids enzymes appeared to be regulated by doxorubicin transcriptionally at 600 nM. In fact many of these channeled sphingolipids into the production of ceramide. However, since the effects of doxorubicin are largely dose-dependent, we wanted to determine whether the effects on sphingolipids enzymes were also dose-dependent. To study that, we performed a qRT-PCR array on the different doses of doxorubicin for the major sphingolipid enzymes (Fig S3). The results demonstrated 3 patterns of regulation: the first showing increases at the low dose of doxorubicin (200-600 nM) and these included sphingomyelin

synthase-2 (SGMS2), acid sphingomyelinase (SMPD1), neutral sphingomyelinase-2 (SMPD3) and alkaline ceramidase-2 (ACER2) (Fig 14 A-D). The second pattern is one of changes at the higher doses (800 and 1000 nM) and these included increases in neutral ceramidase (ASAH2) and decreases in ceramide synthase-4 (CERS4), sphingosine kinase02 (SPHK2) and serine palmitoyl transferase (SPTLC2) (Fig 15 A-D). Finally the last pattern is one of decrease at all doses and that involved ceramide kinase (CERK) (data not shown). A full list of results is shown in Table 1.

A3.3 Lipid profile following doxorubicin mirrors enzymatic changes

Since the transcriptional regulation of the sphingolipid enzymes was dose-dependent and revealed distinct sphingolipid metabolic programs, an analysis of the lipid levels was mirrored to check if these mirrored the changes in the enzymes. Indeed, lipid analysis by LC/MS revealed a biphasic peak of ceramide (at 600 nM and 1000 nM) (Fig 16A). Interestingly, the ceramide species at the 2 doses were different in chain length consistent with the different metabolic programs at these 2 doses (Supplementary figure 4). The 600 nM peak consisted mainly of long chain ceramides while the 1000 nM peak consisted of very long chain ceramides. Sphingosine and sphingosine-1-phosphate showed a dose dependent increase that peaked at 600 nM consistent with the patterns of increase in ACER2 as well as downregulation of SK2 at the higher doses (Fig 16 B-C). No observed changes were noted with dihydrosphingosine (dh-Sph) and dihydrosphingosine-1-phosphate (dh-Sph-1-P) (Supplementary figure 5).

A3.4 Neutral ceramidase: a potential p53 target involved in cell death

As the low dose sphingolipid changes are driven mainly by nSMase2 to mediate a growth arrest phenotype, we sought to determine whether any of the enzymatic changes at the higher doses was responsible for the cell death phenotype seen. Since neutral ceramidase was the only enzyme

upregulated at these doses and as such the most amenable to inhibition, we chose to focus on it. First, immunoblotting analysis for neutral ceramidase revealed a dose-dependent change that mirrored the RNA with increases in protein levels at the higher doses (Fig 17A). Knocking down neutral ceramidase by siRNA resulted in increase in cell number as determined by Trypan Blue counts suggesting a role for this enzyme in doxorubicin-mediated cell death (Fig 17B). Finally, as part of an effort to determine which sphingolipid enzyme changes are dependent on p53, a qRT PCR array was performed to look at the enzymatic changes following WT p53 overexpression in MCF-7 cells. Five of the sphingolipid enzymes were upregulated and neutral ceramidase was among those suggesting that this enzyme is a transcriptional target of p53. (data not shown)

A4. Discussion:

This study demonstrates the dose-dependent effect of doxorubicin on sphingolipids. It reveals enzymatic as well as lipid signatures at each dose, associated with different biologies. A major finding of the study is the different sphingolipid metabolic programs at each dose. Interestingly, at sublethal doses, doxorubicin treatment is associated with activation of enzymes that promote growth arrest and survival. Interestingly, in that category, we find nSMase2 and ACER2 being the major enzymes activated with increases in medium and long chain ceramides as well as sphingosine and S-1-P. At higher doses, we find upregulation of neutral ceramidase as well as downregulation of sphingosine kinase 2. This downregulation is mirrored by an increase in ceramide species. However, the species involved are different and comprise mainly the long and very long species. The biological effects of these differential changes remain to be studied. The lower doses increases in ceramide, sphingosine and S1P and their mirroring of the upregulation of nSMase2 and ACER2 suggest that manipulation of these enzymes (inhibiting, overexpressing) could provide insight to the function of the lipid. At higher doses, the changes are as not clear cut

and whether there is an increase in flux along the *de novo* pathway remain to be seen. As such, the pathway of ceramide generation at these doses as well as their potential involvement in cell death needs to be assessed.

Another finding from this study is the potential role of neutral ceramidase in mediating cell death following high doses of doxorubicin. Interestingly, the upregulation of neutral ceramidase is not associated with increase in sphingosine or S1P at the high doses. One hypothesis to be tested is that neutral ceramidase is activating its reverse activity to generate ceramide at these high doses of doxorubicin. Indeed the reverse activity of neutral ceramidase has been previously reported (383) and has been associated with increase in ceramide. Interestingly, the fact that neutral ceramidase is activated by p53 and that p53 mediates doxorubicin-dependent cell death suggest that neutral ceramidase could be a downstream target of p53 in response to doxorubicin that generates ceramide and activate apoptosis.

Chapter 4

Nuclear C18:1 ceramide mediates nSMase2-dependent growth arrest via the B55/cohesin axis

4.1 Introduction:

Ceramide is a bioactive sphingolipid that mediates numerous biological functions such as growth arrest and apoptosis (1,2). Recent literature started to focus on the compartment specific distribution of ceramide as well as the specific chain length that mediate biological functions (2). From this, three distinct pools of ceramide emerged: the endoplasmic reticulum (ER) pool which is generated through *de novo* synthesis and has been implicated mainly in pro-apoptotic functions (362,384-387), the inner plasma membrane pool generated by neutral sphingomyelinase activity (20) and has been implicated in growth arrest (58,63), apoptotic (114) as well as differentiation functions (221), and finally the lysosomal pool which has a myriad of fucntions including regulation of cytokines and cancer invasion and metastasis (388,389). Interestingly, the presence of ceramide has been reported in other cellular organelles such as the mitochondria and the nucleus (31,36). The generation of these nuclear pools of ceramides as well as their metabolism is not well understood and of all the sphingolipid enzymes, only few have been reported to localize to the nucleus.

Neutral sphingomyelinase-2 is a ceramide-generating enzyme located in the golgi, as well as in the inner leaflet of the plasma membrane (20,93). Among its many functions, it has been implicated in mediating growth arrest mainly in response to confluence and All-Trans Retinoic Acid (ATRA) (58,63), and more recently the chemotherapeutic agent doxorubicin. While confluence and ATRA appear to induce an nSMase2-dependent G_0/G_1 arrest that are dependent on phosphorylation of catenin and S6K respectively (59,63), the mechanism of nSMase2-mediated growth arrest in response to doxorubicin is not understood.

Doxorubicin is a DNA damaging agent that results in the generation of both ssDNa as well as dsDNA breaks (390). This can result in the activation of both S and G2 checkpoints. The S phase checkpoint is transient in nature and depends mostly on the activation of an ATR, Chk1 axis (228). Chk1 is thought to eventually phosphorylate the phosphatase Cdc25a which targets it to degradation and prevent it from removing the inhibitory phosphorylation on Cdc2 which in turn results in the inability of the Cdc2/cyclin A or Cdc2/cyclin E complexes from mediating progressing through S phase and as such activates the checkpoint (391). The role of p53 in regulating the S phase checkpoint has been unclear until it was reported that an isoform of p53, delta p53 (δp53), is a mediator of ATR-controlled S phase checkpoint (392). In fact, this isoform is specifically activated during S phase, mediates p21 induction to transcriptionally repress cyclin A, cyclin E and cdc2 (392).

In this study, we demonstrate that nSMase2 is upregulated following treatment with agents that cause ssDNA breaks. This upregulation is transcriptional and is important for mediating protection form apoptosis following DNA damage. This is achieved through an nSMase2-dependent prolongation of the S phase checkpoint. Mechanistically, nSMase2 appears to localize to nuclear speckles following doxorubicin treatment and produces nuclear C18:1 ceramide that mediates this protection. In turn, the B55 subunit of PP2A appears to be a downstream target of nSMase2 in order to regulate the phosphorylation of the cohesion complex proteins, namely rad21. This work establishes a role for the bioactive lipid C18:1 ceramide in mediating a protective role following doxorubicin treatment,

4.2 Materials and methods:

4.2.1 Materials

MCF7 breast carcinoma cells were obtained from ATCC (Manassas, VA). RPMI culture medium, fetal bovine serum and SuperScript reverse transcriptase were obtained from Invitrogen. Antibodies for nSMase2 (H195), Cyclin A and SC35 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for PARP, p-cdc2, p-Rb, histone H3, Na+/K+ ATPase and IKK alpha were from Cell Signaling Technologies (Beverly, MA). The inhibitor ZVAD-FMK was from Selleck Chem (Boston, MA). The chemicals doxorubicin, camptothecin, 5-fluorouracil, taxol and etoposide were from Sigma. siRNA for nSMase2, p53 and B55were from Life Technologies (Grand Island, NY). Porcine brain sphingomyelin and phosphatidylserine were from Avanti Polar Lipids (Alabaster, AL).

4.2.2 Cell culture and siRNA

MCF-7 cells were grown at 37 °C, 5% CO2 in 10% fetal bovine serum in RPMI. Cells were subcultured in 60-mm dishes (175, 000 cells) and medium was changed 1 hour before the start of experiments. For siRNA experiments, cells were plated in 60-mm dishes (100, 000 cells) and 24 hours later transfected with 10 nM negative control or 10 nM of the desired siRNA using Lipofectamine RNAiMAx reagent (Life Technologies) according to manufacturer's protocol. After 24 hours, cells were incubated in fresh medium for 1 hour prior to treatment.

4.2.3 Protein extraction and immunoblotting

For cellular protein extraction, cells were scraped in 0.75% SDS and lysed by sonication. Bradford reagent (Bio-Rad) was used to determine protein concentration prior to immunoblotting. Lysates were mixed with equal volumes of 2x Laemelli buffer (Bio-Rad) and boiled for 5 minutes.

The protein was separated by SDS-PAGE using the Criterion gels (Bio-Rad) and immunoblotted as described previously (63).

4.2.4 Real Time PCR

RNA extraction was performed using the Purelink RNA kit (Life Technologies) according to manufacturer's protocol. RNA quality and concentration was verified by nanodrop after which 1 μg of RNA was transformed into cDNA using the Quanta cdna kit according to manufacturer's protocol. For qRT PCR, reactions were run in triplicates in 96 well plates with each reaction containing 10 uL of 2X iTAQ mastermix, 5 uL of cdna, 1 uL of taqman primer-probe and 4 uL of water. The primer-probes used were purchased from life technologies and amplified nSMase2 (Cat# 4331182), Actin, (Cat# 4448484) and CREB3L1 (Cat# 4331182).

4.2.5 Neutral Sphingomyelinase Assay

Neutral sphingomyelinase activity was assayed as described previously using ^{14}C-[methyl]sphingomyelin as substrate (63). Briefly, SM from bovine brain (Avanti polar lipids) and C14 PS (Avanti polar lipids) were dried under N2(g) and resuspended in Triton X-100 mixed micelles by sonication. The final reaction conditions contained 0.1% Triton X-100, 100 mM Tris buffer, pH 7.5, 20 mM $MgCl_2$, and 5 mM DTT with 5 mol% SM (SM only) or 5 mol% SM + 5 mol% PS (SM + PS). Cellular lysates containing 80 μg of protein were added to 100 μL of assay buffer and were incubated for an hour. After that, reaction was quenched and a modified Bligh and Dyer extraction was performed. The aqueous phase (700 μL of supernatant) was transferred to scintillation vials containing 3 mL of scintillation fluid and counted.

4.2.6 Analysis of Cellular Sphingolipids

Prior to collection, cells were incubated in serum-free medium (RPMI with 0.1% fatty-acid free BSA) for 3 hours. Cells were scraped and pelleted and extraction and analysis by LC/MS mass spectrometry was performed as described previously (63). Lipids were normalized to total phosphate levels of selected sample.

4.2.7 Cell cycle analysis by flow cytometry

Cells were collected and washed 2x with PBS then fixed with 1 mL of 70% ethanol overnight. The next day, cells were pelleted, ethanol removed and washed twice with PBS and incubated with 0.5 mL of PI/RNase solution (cell signaling) supplemented with 0.2% Triton X-100 for 1 hour. Cells were analyzed on a FACSCalibur machine and data were analayzed using the ModFit software

4.2.8 MTT assays

Cells were seeded in 6 well plates. Following the end of the experiment, media was aspirated and cells were incubated in 1 mL of media and 1 mL of MTT reagent at a concentration of 5 mg/mL for 30 minutes. Media was then aspirated and 2 mL of DMSO were added for 5 minutes after which 200 μL aliquots were taken in duplicates and absorbance was measured at 570 nm.

4.2.9 Phosphoproteomics

Lysis, Tryptic Digestion, iTRAQ Labeling – Pelleted cells were lysed with 100ul of lysis buffer (50mM HEPES, 0.5mM EDTA, 1% NP-40, 0.1% SDS at pH 8). 1ul each of protease inhibitor cocktail 1 (Sigma-Aldrich), phosphatase inhibitor cocktail 2 (Sigma-Aldrich), and phosphatase inhibitor cocktail 3 (Sigma-Aldrich) were added to each sample. The lysate was sonicated for 30seconds and aspirated through a 25gauge needle five times. The lysate was then centrifuged at 14,000rpms for 5 minutes at 4°C to pellet any insoluble material. The supernatant

was then removed and placed into a fresh 1.5mL centrifuge tube and a BCA assay (Pierce) performed on 10uLof lysate to determine protein concentration. 160ug of each sample were brought up to 40uL final volume with 100mM triethylammonium bicarbonate buffer (TEAB) pH 7.8. *Tris*(2-carboxyethyl)phosphine (TCEP) was added to a final concentration of 5mM, the samples heated to 55°C for 20min, then allowed to cool to room temperature. Methyl methanethiosulfonate (MMTS) was added to a final concentration of 10mM and the samples incubated at room temperature for a further 20 min to complete blocking of free sulfhydryl groups. Proteins were then precipitated by the addition of 4x sample volume of methanol, followed by 2x sample volume of chloroform and 3x volume of water. The samples were then incubated at -20°C for 2 hours and centrifuged at 14,000 for 10mins at 4°C. The upper liquid layer was removed and discarded without disturbing the pellet. Methanol was added to 3x the original sample volume, the sample vortexed and then centrifuged at 14,000rpms for 10mins at 4°C. The entire supernatant was removed and discarded without disturbing the pellet, and the pellet was allowed to dry. 2ug of sequencing grade trypsin (Promega) was then added to the samples and they were digested overnight at 37°C and dried in vacuo. Peptides were reconstituted in 50uL of 0.5M TEAB/70% isopropanol and labeled with 8-plex iTRAQ reagent for 2 hours at room temperature essentially according to Ross et al (2004). Labeled samples were then acidified to pH 4 using formic acid, combined and concentrated in vacuo until ~10uL remained.

Phosphopeptide Enrichment – 125uL of wash solution (0.1% TFA:50% Acetonitrile) was added to TiO2 resin. The slurry was vortexed and spun down and the supernatant was removed. This wash was repeated once more. 100uL of binding solution (1 M lactic acid in 0.1% TFA:50% Acetonitrile) was added to the beads. 60uL of binding solution was then added to the

sample and it was vortexed. The entire sample was added to the beads. The tube containing the TiO2 resin and the sample was vortexed for 45 minutes. After vortexing the sample was centrifuged at max speed (14,000rpms) for 10 mins and the supernatant was removed. The supernatant was saved for later us as the "flow-through". The TiO2 resin was washed 3 times with wash solution. 25uL of elution solution (50mM KH2PO4, pH to 10.5 with ammonia) was added to the resin and the sample was allowed to stand for 5 minutes before being vortexed and centrifuged at max speed for 5mins. The supernatant was added to 25uL of neutralization solution (5% Formic acid:50% Acetonitrile) in a clean tube. The elution step was repeated again and both elutions were combined.

2-Dimensional Fractionation – Peptides were fractionated using a high-low pH reverse phase separation strategy adapted from Gillar et al. (2005). For the first (high pH) dimension, peptides were fractionated on a 10cm x 1.0mm column packed with Gemini 3u C18 resin (Phenomenex, Ventura, CA) at a flow rate of 100ul/min. Mobile phase A consisted of 20mM ammonium formate pH 10 and mobile phase B consisted of 90% acetonitrile/20mM ammonium formate pH 10. 100 ug of total peptide was reconstituted with 50uL of mobile phase A and the entire sample injected onto the column. Peptides were separated using a 35 minute linear gradient from 5% B to 70% B and then increasing mobile phase to 95% B for 10 minutes. Fractions were collected every minute for 80 minutes and were then combined into 22 fractions using the concatenation strategy described by Wang et al. (2011). An estimated 1ug of peptide from each of the 22 fractions was then separately injected into the mass spectrometer using capillary reverse phase LC at low pH, described below.

Capillary LC Mass Spectrometry - An Orbitrap Velos Pro mass spectrometer (Thermo Scientific), equipped with a nano-ion spray source was coupled to an EASY-nLC system

(Thermo Scientific). The nano-flow LC system was configured with a 180-µm id fused silica capillary trap column containing 3 cm of Aqua 5-µm C18 material (Phenomenex), and a self-pack PicoFrit™ 100-µm analytical column with an 8-µm emitter (New Objective, Woburn, MA) packed to 15cm with Aqua 3-µm C18 material (Phenomenex). Mobile phase A consisted of 2% acetonitrile/0.1% formic acid and mobile phase B consisted of 90% acetonitrile/ 0.1% formic Acid. 3uL of each sample dissolved in mobile phase A, was injected through the autosampler onto the trap column. Peptides were then separated using the following linear gradient steps at a flow rate of 400 nL/min: 5% B for 1 min, 5% B to 35% B over 70 min, 35% B to 75% B over 15 min, held at 75% B for 8 min, 75% B to 8% B over 1 min and the final 5 min held at 8% B.

Eluted peptides were directly electrosprayed into the Orbitrap Velos Pro mass spectrometer with the application of a distal 2.3 kV spray voltage and a capillary temperature of 275°C. Each full-scan mass spectrum (Res=60,000; 380-1700 *m/z*) was followed by MS/MS spectra for the top 12 masses. High-energy collisional dissociation (HCD) was used with the normalized collision energy set to 35 for fragmentation, the isolation width set to 1.2 and activation time of 0.1. A duration of 30 seconds was set for the dynamic exclusion with an exclusion list size of 500, repeat count of 1 and exclusion mass width of 10ppm. We used monoisotopic precursor selection for charge states 2+ and greater, and all data were acquired in profile mode.

Database Searching - Peaklist files were generated by Mascot Distiller (Matrix Science). Protein identification and quantification was carried using Mascot 2.4 (Perkins et al., 1999) against the Uniprot Human sequence database (89,706 sequences; 35,609,686 residues). Methylthiolation of cysteine and N-terminal and lysine iTRAQ modifications were set as fixed modifications, methionine oxidation and deamidation (NQ) as variable. Trypsin was used as

cleavage enzyme with one missed cleavage allowed. Mass tolerance was set at 30 ppm for intact peptide mass and 0.3 Da for fragment ions. Search results were rescored to give a final 1% FDR using a randomized version of the same Uniprot Human database. Protein-level iTRAQ ratios were calculated as intensity weighted, using only peptides with expectation values < 0.05. Global ratio normalization (summed) was applied across all iTRAQ channels. Protein enrichment was then calculating by dividing sample protein ratios by the corresponding control sample channel.

4.2.10 Generation of bacterial sphingomyelinase and bacterial ceramidase constructs

Tagging bSMase with 3xNLS: bSMase was amplified with primers F: GCctgcagGAAGCATCTACAAATCAAAATG and R: CGCctcgagCTTCATAGAAATAGTCGCCTC, digested, gel purified and cloned into the Pst1/Xho1sites of pCMV/myc/nuc, pShooter vector from Life Technologies cat#V821-20.

Tagging bSMase-3xNLS with V5: bSMase - 3xNLS was amplified with primers F: gcGGATCCgccaccATGGCCCAGGTGCAGCTGCAG and R: GCtctagaGCtgcggccccattcagatcctcttc, digested, gel purified and cloned into the BamHI / Xba1 sites of pEF6/V5-His-TOPO vector from Life Technologies, cat# K9610-20

Tagging bSMase with ER signal peptide and ER retention signal: bSMase was amplified w/ primers F: GCctgcagGAAGCATCTACAAATCAAAATG and R: CGCctcgagCTTCATAGAAATAGTCGCCTC, digested, gel purified and cloned into the Pst1/Xho1sites of pCMV/myc/ER, pShooter vector from Life Technologies cat#V823-20.

Tagging bSMase-ER w/ V5: bSMase – ER was amplified with primers F: gcGGATCCgccaccATGGGATGGAGCTGTATCATC and R:

gcTCTAGAgcCAGCTCGTCCTTCTCGCTTGCGGC, digested, gel purified and cloned into the BamHI / Xba1 sites of pEF6/V5-His-TOPO vector from Life Technologies, cat# K9610-20

Tagging bCDase with 3xNLS: bCDase was amplified with primers F: gcCTGCAGctgccctaccgcttcggcctgg and R: cgcCTCGAGgggagtggtgccgagcacctcg, digested, gel purified and cloned into the Pst1/Xho1sites of pCMV/myc/nuc, pShooter vector from Life Technologies cat#V821-20.

Tagging bSMase-3xNLS w/ V5: bCDase - 3xNLS was amplified with primers F: gcGGTACCgccaccATGGCCCAGGTGCAGCTGCAG and R: gcTCTAGAgcTACCTTTCTCTTCTTTTTTGGATC, digested, gel purified and cloned into the KpnI / Xba1 sites of pEF6/V5-His-TOPO vector from Life Technologies, cat# K9610-20

4.2.11 Statistical analysis

All experiments are n of 3 unless expressed otherwise. Bars are representative of means and standard deviation. Two-way ANOVA was employed for the analysis of samples with multiple variables (for example treatment and siRNA) and one-way ANOVA was employed for the rest. Significance is achieved at $p<0.05$

4.3 Results:

4.3.1 nSMase2 upregulation is specific to ssDNA breaks generating agents

Previous published data from our lab showed that nSMase2 upregulation following doxorubicin treatment is transcriptional and depends on the ATR/Chk1/p53 axis. The activation of this axis is dependent on single stranded DNA breaks (ssDNA) generation. Therefore, we sought to determine whether nSMase2 upregulation is a general mechanism following formation of

ssDNA breaks. Using MCF-7 breast cancer cells, a panel of agents were tested for their ability to induce NSMase activity. Indeed, only doxorubicin, camptothecin, ultra-violet (UV) radiationwere able to increase NSMase activity (Fig 18A and supplemental Fig S6). Of these agents, UV is a potent generator of ssDNA breaks, camptothecin and doxorubicin are potent generators of both ssDNA and dsDNA breaks. Next, we wanted to determine if the increase in NSMase activity was concomitant with an increase in nSMase2 protein levels. As can be seen, camptothecin was able to induce a dose-dependent increase in nSMase2 protein levels, similar to doxorubicin (Fig 18B). This is consistant with the hypothesis that ssDNA breaks generation is the lesion responsible for the upregulation of nSMase2. As doxorubicin effects on nSMase2 are transcriptional, we sought to determine whether camptothecin and UV both upregulated nSMase2 transcriptionally. Indeed, treatment with these agents was found to increase nSMase2 transcriptionally (Fig 18C and Fig. S7), in a p53-dependent manner (Fig 18 D-E). Taken together, these results suggest that nSMase2 activated transcriptionally following ssDNA breaks generation.

4.3.2 nSMase2 upregulation protects from apoptosis following ssDNA breaks

Previously, our lab has shown that nSMase2 transcriptional induction is necessary for doxorubicin-induced growth arrest. The ability of the cancer cell to induce growth arrest following genotoxic chemotherapy has been linked to its chemoresistance, as growth arrest allows the cell to repair the DNA damage before proceeding with replication. In many instances, blocking the ability of the cancer cell to induce growth arrest resulted in increased chemosensitivity. As such, we wanted to determine if knockdown of nSMase2 sensitized the cells to chemotherapy. Knocking down nSMase2 resulted in decreased MTT readings at 48 and 72 hours following doxorubicin (Fig 19A) and camptothecin (Fig 19B) treatments. This suggested a lower cell survival in the nSMase2 knockdown population treated with doxorubicin. To confirm that, ZVAD-FMK, a pan-caspase

inhibitor was added and blocked the decrease in MTT readings following nSMase2 knockdown suggesting that this population was apoptosing (Fig 19C) Biochemically, this was associated with an increase in PARP cleavage. (Fig 19D). Taken together, these data point to the importance of nSMase2 in mediating chemoresistance to ssDNA break generating agents in general, and topoisomerase poisons in particular.

4.3.3 nSMase2 prolongs the duration of S phase

To study the mechanism of nSMase2-mediated growth arrest, a cell cycle analysis by flow cytometry was performed. Results indicated that at 24 hours post-treatment, nSMase2 knockdown cells did not exhibit an increased in S phase population seen with cells treated with doxorubicin (Fig 20A). These results could be explained by two hypothesis; the first is that cells without nSMase2 do not arrest in S phase while the second is that they do but they recover at an earlier time, given the transient nature of the S phase checkpoint. To differentiate, a time course was performed and showed that cells without nSMase2 recover faster from S phase arrest following doxorubicin than control cells (Fig 20B). Interestingly, inhibition or mutation of regulators of S phase are usually associated with radioresistant DNA synthesis. As such, the ability of nSMase2 to control radioresistant DNA synthesis was evaluated. Indeed nSMase2 knockdown was associated with doubling of RDS in comparison to control (Fig 20C). Looking at the regulators of the S phase checkpoint, knocking down nSMase2 did not affect the levels of cyclin A, cdc2 or its phosphorylation (Fig 20D). Since nSMase2 expression is controlled by p53, p53 targets involved in cell cycle arrest were evaluated and nSMase2 knockdown did not alter levels of p21 nor Rb (Fig 20D). These results suggest that nSMase2 is involved in exit from S phase.

4.3.4 nSMase2 is localized to the nucleus following doxorubicin treatment

Cell cycle control is achieved mostly by nuclear proteins. This is in stark contrast to the localization of nSMase2 which is reported to be in the golgi, as well as at the inner leaflet of the plasma membrane. However, these studies were done on overexpressed proteins as the lack of a good antibody hindered the studies of the endogenous protein. In order to study the localization of nSMase2 following doxorubicin treatment, a monoclonal antibody was raised against the C-terminus (see materials and methods) and verified for its ability to recognize nSMase2 (Fig. S8). Immunofluorescence studies with that antibody showed nSMase2 to localize to the nucleus after doxorubicin treatment (Fig 21A). Furthermore, this was confirmed by cellular fractionation (Fig 22B). Activity assays revealed an increase in the nuclear NSMase activity (Fig 22C), that was phosphatidylserine-dependent (Fig 21D and Fig S9A-C), and inhibited by knockdown of nSMase2 (Fig 21E and Fig S9D-F). Looking at lipids, C14 and C18:1 ceramide were found to be selectively upregulated in the nuclear fractions (Fig 21F and Fig. S10 and S11). Finally, the localization of nSMase2 staining in the nucleus indicated distinct clustering between regions of chromatin. As such, co-staining was performed with SC35, a marker of nuclear speckles. Speckles are interchromatin granule clusters which have been shown to contain phospholipids and phospholipid metabolism enzymes. Co-staining with SC35 and nSMase2 revealed distinct regions of overlap suggesting that nSMase2 localizes, at least partially, to nuclear speckles (Fig 21G). Collectively, these data demonstrate nSMase2 to be a nuclear speckles enzyme following doxorubicin treatment.

4.3.5 Nuclear C18:1 ceramide is the lipid responsible for nSMase2 protective *biology*

The biological effect of nSMase2 upregulation following doxorubicin treatment appears to be dependent on its catalytic activity. To confirm that, overexpression of a nuclear targeted sphingomyelinase cloned from *Bacillus cereus* (bSMase) was performed (Fig S12). This overexpression resulted in the rescue of the phenotype seen following nSMase2 knockdown (Fig

22A). As a control, overexpression of ER targeted bSMase was performed and did not result in rescue suggesting the importance of the localization of the ceramide produced. Interestingly, the overexpression of Nuc-bSMase resulted in significant changes with the highest in terms of fold change being C18:1 ceramide (Fig S13). To further validate that the effect is dependent on ceramide and not any downstream lipid, a co-expression of nuclear bSMase and bCDase was performed. As can be seen, while expression of Nuc-bSMase was able to rescue, the expression of Nuc-bCDase as well as co-expression of both did not (Fig 22B). This suggests that the effect is dependent on nuclear ceramide. To further narrow it down to the ceramide specie, fatty acid supplementation experiments were performed using myristate (C14), palmitate (C16) and oleate (C18:1) as both C14 and C18:1 were found in the nuclear fraction upon doxorubicin treatment. Interestingly only treatment with oleate was able to rescue the phenotype exhibited in the nSMase2 knockdown cells (Fig 22C) and fumonisin B1 pre-treatment blocked that effect (Fig 22D). Taken together, these results suggest that nuclear C18:1 is the effector lipid mediating nSMase2 biology.

4.3.6 The B55 subunit of PP2A is a potential target of nSMase2 in response to doxorubicin.

The yeast ortholog of nSMase2 activates a protective G2/M arrest in response to hydroxyurea through C18:1 phytoceramide production. Moreover, CDC55, a subunit of PP2A, was implicated downstream of Isc1. As such, we looked at the mammalian homolog of CDC55, B55. As can be seen, B55 knockdown followed by treatment with doxorubicin phenocopies nSMase2 knockdown (Fig 23A). Furthermore, immunofluorescence reveals B55 to localize to nuclear speckles, both at baseline and after treatment with doxorubicin (Fig 23B). As this suggested the involvement of B55 downstream of nSMase2, we looked at possible dephosphorylation targets downstream of nSMase2 through a phosphor screen. As can be seen, 100 hits were obtained and were analyze by DAVID software for biological processes (Fig 23C)

and cellular localization (Fig 23D). Interestingly, cell cycle regulation was one of the major processes found to be regulated. Moreover, more than 80% of the targets were nuclear confirming the localization of nSMase2. Taken together, these data suggest that B55 is a downstream target of nSMase2 that regulates protein phosphorylation status involved in nSMase2 biology

4.4 Discussion:

This study demonstrates the role of nSMase2 in mediating a protective S phase checkpoint in response to topoisomerase inhibitors. We find that this effect is due to nuclear nSMase2 production of C18:1 ceramide and its subsequent regulation of the B55 subunit of PP2A. These data provide novel insights into the effector role of nuclear C18:1 ceramide in regulating the S phase checkpoint function downstream of p53.

A major finding of this study is the protective function of C18:1 ceramide in the nucleus. Ceramide presence in the nucleus has previously been reported in rat liver nuclei after injury to mediate hepatocyte apoptosis (393,394). Furthermore, staining with anti-ceramide antibodies has revealed the presence of ceramide in the nucleus of HL-60 cells following doxorubicin treatment as well as in Jurkat cells following Fas treatment (358,395). On the other hand, yeast studies with the ΔIsc1 strain in *S. cervisiae* have revealed its sensitivity to hydroxyurea (396). Interestingly this was mediated by C18:1 phytoceramide, as supplementation of the media with oleate was sufficient to rescue the sensitivity of ΔIsc1 to HU (397). Our findings in MCF-7 cells reconciliate the yeast results with some of the literature on nuclear ceramides. In its implication it suggests an evolutionary role for C18:1 ceramide in mediating protection against DNA damage whether through direct strand breaks or through ribonucleotide reductase inhibitors. It remains to be seen whether Isc1 is a nuclear enzyme following hydroxyurea treatment and whether as a consequence

the C18:1 phytoceramide is nuclear. From an importance point of view, our findings constitute a departure from the traditional point of view that ceramide is always associated with pro-death phenotypes. Certainly, the endoplasmic reticulum generated ceramide mediates many of the pro-apoptotic functions attributed to ceramide and this was further seen in this study when overexpression of ER targeted bacterial sphingomyelinase caused cell death. However, the nuclear species of ceramide produced appear to have a protective role. Further studies are needed to characterize these nuclear pools of ceramide, their generation and the enzymes responsible for their catabolism.

From an enzymatic point of view, an important finding is the novel localization of nSMase2 to nuclear speckles. Most of the studies done on nSMase2 localization have utilized overexpression systems due to the lack of efficient molecular tools to study its endogenous localization. As such nSMase2 localizes to the Golgi apparatus (93) and to the plasma membrane following certain stimuli such as confluence and TNF-α (58,102). The 2 hydrophobic segments on its amino terminus as well as lysine palmitoylations mediate its insertion on the inner leaflet of the plasma membrane (84,92). Previous studies have reported the presence of neutral sphingomyelinase activity in the nucleus (394). Furthermore, nSMase1 was shown to be mostly a nuclear enzyme following through immunohistochemistry and cellular fractionation studies (32). However, this is the first report of the localization of nSMase2 to the nucleus. Questions remain as to how this localization happens. Our data suggest that it is possibly through an unidentified protein-protein interaction where nSMase2 piggybacks on another protein bound to the nucleus. This is a hypothesis of exclusion at this point as nSMase2 cannot passively diffuse through nuclear pores and cloning of the only putative NLS, a stretch of basic amino acids, into a GFP vector did not increase the localization of GFP to the nucleus (Fig S14).

This study also provide new insight into the mechanisms governing the S phase checkpoint. We describe a new mechanism downstream of p53 and independent of the known cyclins and Cdks that regulate S phase. It involved the activation of the B55 subunit of PP2A and the regulation of the cohesin complex. Previously, phosphorylation changes on the cohesion complex have been implicated in the intra S phase checkpoint. Most of these have been described on Smc3 (325). However, since the cohesion is a multimeric protein complex, it is easy to imagine that phosphorylation changes during a specific biology would not be limited to one component of the protein complex. On the other hand, a role for the B55 subunit of PP2A in S phase arrest has not been described previously. The connection between nSMase2 and the B55 subunit of PP2A as well as the connection between B55 and the cohesion complex is still not well characterized and needs further studies to fully understand the signaling cascade downstream of nSMase2.

Appendix B

Dasatinib inhibits doxorubicin-induced invasion at sublethal doses

B1. Introduction:

Doxorubicin is an anthracycline used in the treatments of many tumors (398,399). Among those, doxorubicin is used in many chemotherapeutic regimens for the treatments of primary as well as metastatic breast cancer (400-402). Its anti-tumor activity is mainly linked to its ability to induce DNA damage, whether through ssDNA breaks or dsDNA breaks, as well as its ability to generate reactive oxygen species (403). However, there is conflicting literature on whether doxorubicin possesses anti-invasive properties. In the initial dose escalation clinical trials, it was noticed that low concentrations of doxorubicin was associated with higher incidence of local recurrence after surgery, rather than distant metastasis suggesting increased local invasion (350). Subsequent cellular studies have revealed cell-type dependent effects of doxorubicin on tumor invasiveness. While aggressive breast cancer models such as xenografted MDA-MB-231 revealed anti-invasive properties of doxorubicin (404,405), less aggressive models such as murine 4T1 tumors showed that doxorubicin increased invasion (406)

Dasatinib is an ATP competitive inhibitor of the Src family of protein kinases (407). It has been used as a second line treatment for chronic myelogenous leukemia, after failure of imatinib (408). Recent evidence however suggests its superiority to imatinib in the treatment of CML (409,410). In breast cancer, dasatinib has had limited success in pre-clinical models as well as phase I/II studies. However, this might be due to the failure to identify specific subsets of patients which might benefit from the use of this inhibitor. As such, the need to understand the molecular mechanisms that control tumor development and progression is necessary in breast cancer.

In this study, we establish that sublethal doxorubicin induces transcriptional upregulation of invasive pathways in MCF-7 cells. This is confirmed as both doxorubicin and camptothecin

activate Src. Activation of Src in conjunction with chemotherapy is associated with a worse prognosis. Indeed sublethal doxorubicin induces an increase in MCF-7 tumor cell migration that is inhibited by Dasatinib.

B2. Results

B2.1 Sublethal doxorubicin activates invasive pathways

Studies on the cancer invasiveness effects of doxorubicin are mostly contradictory and conducted on highly metastatic breast cancer cell lines. In this study, we assessed the effects of doxorubicin on the non-invasive breast cancer cell line MCF-7. Doxorubicin induces a dose-dependent changes in cellular morphology. Sublethal doxorubicin (600 nM) or less is associated with increased invasive morphological changes such as spindle cellular morphology as well as cellular protrusions (Fig 24A-B). RNA sequencing analysis of genes that are selectively upregulated by doxorubicin transcriptionally revealed not only transcriptional upregulation of DNA damage pathways but also those of invasive pathways (Fig 24C and Fig S15). These pathways appear to converge on Src, a known protein kinase involved in invasion (supplementary figure S15). Interestingly, Kaplan Meier plots of breast cancer patients with low and high expression of Src revealed that in the subset of patients who received chemotherapy, high expression of Src was associated with a poor prognosis. In comparison, in patients that did not receive chemotherapy, expression of Src did not affect survival (Fig 24D). These results point to an invasive pathway that is upregulated by doxorubicin at sublethal doses.

B2.2 Sublethal doxorubicin activates Src in a p53-dependent manner to induce migration

Following data from RNA sequencing, we set out to determine if Src was activated by doxorubicin at sublethal doses. As can be seen, Src is activated at doses of 200-800 nM as

evidenced by immunoblotting with the pY416 Src antibody, a marker of activated Src (Fig 25A). This is accompanied by increases in Src protein levels and mRNA (Fig 25 A-B). Interestingly, the activation of Src is time-dependent and peaks at later time points (24 and 48 hours) (Fig 25 C). Since p53 has been implicated in the regulation of Src, we sought to determine whether p53 and by extension DNA damage is responsible for Src induction. As can be seen knockdown of p53 by siRNA results in the abrogation of doxorubicin-dependent Src induction (Fig 25D). Finally, we sought to determine whether doxorubicin was inducing Src-dependent cellular migration of MCF-7 cells. Using 2D collagen matrix assays for migration, doxorubicin was able to induce migration at sublethal doses. This effect was prevented by the Src inhibitor, Dasatinib suggesting an essential role for Src in doxorubicin-induced migration (fig 26). Taken together, these results suggest an activation of a Src-dependent pathway of migration at sublethal dose controlled by p53.

Chapter 5

Concluding discussion

5.1 Summary

This thesis explored the role of nSMase2 in the DNA damage response generally, and more particularly in response to topoisomerase inhibitors, namely doxorubicin. We provided evidence that nSMase2 is activated transcriptionally by topoisomerase inhibitors, doxorubicin and camptothecin, as well as UV. Doxorubicin activation of nSMase2 is dose dependent, peaking at the sublethal dose 600 nM, and is associated with an increase in NSMase activity as well as production of ceramide. The transcriptional regulators of this induction are the DNA damage proteins ATR, Chk1 and p53. Furthermore, overexpression of p53 in absence of DNA damage is sufficient by itself to induce nSMase2 transcriptionally, albeit not to the same extent as with doxorubicin

The function of nSMase2 in response to doxorubicin was found to involve mediating its growth arrest effect. In fact, doxorubicin treatments results in the activation of an S and a G2 checkpoint, the former being dependent on nSMase2 induction. As such, cells with nSMase2 knockdown recovered from S phase faster than WT control cells suggesting a role for nSMase2 in regulating exit from S phase. This is a protective function as nSMase2 knockdown sensitizes cells to doxorubicin and is associated with an increase in DNA damage as well as induction of apoptosis.

Discrepancy arose as nSMase2 is primarily a plasma membrane protein that is apparently mediating a nuclear event. As such, the localization of nSMase2 following doxorubicin treatment was assessed using a newly generated monoclonal antibody and was found to be nuclear. This was confirmed by cellular fractionations, activity assays and lipid analysis. Moreover, nSMase2 antibody showed co-staining with SC35, a marker of nuclear speckles, by immunoflurorescence. These speckles are subnuclear compartments involved in cell cycle regulation as well as RNA

splicing and known to contain phospholipids and their metabolizing enzymes. Overexpression of a nuclear targeted bSMase rescued the sensitization induced by knockdown of nSMase2 as did C18:1 fatty acid supplementation. This suggested that nuclear C18:1 ceramide is the lipid responsible for the biology observed with nSMase2.

Mechanistically, the protective effect of nSMase2 in response to doxorubicin were similar to the protective effect of its yeast ortholog Isc1 in response to hydroxyurea. In the latter case, Isc1 signals through activation of CDC55, a protein phosphatase. Knockdown of the B55 subunit of PP2A, the mammalian homolog of CDC55 phenocopied nSMase2 knockdown. Furthermore, a phosphoproteomics screen was carried out to determine possible phospho-targets downstream of CDC55 and revealed regulation of the cohesion complex dephosphorylation by nSMase2.

The changes in nSMase2 transcriptional levels are not isolated, but part of dose-dependent transcriptional changes of sphingolipid enzymes with doxorubicin. Studying these changes, two patterns clearly emerge; one at low sublethal doses which involve mainly nSMase2 and alkaline ceramidase 2 and is associated with an increase in long chain ceramides, sphingosine and S1P. The other is changes at the high doses involving mainly neutral ceramidase and sphingosine kinase 2 and is associated with an increase in very long chain ceramides.

In parallel with the cell cycle role of nSMase2 in response to doxorubicin, nSMase2 regulates Src activation following sub-lethal doxorubicin treatment. RNA sequencing of doxorubicin treated cells at 600 nM revealed transcriptional upregulation of invasive pathways centered around Src. Further verifications revealed that indeed Src is transcriptionally induced as well as activated in response to doxorubicin and camptothecin. This activation appeared to depend on nSMase2 as its knockdown abolished the increase in Src. Functionally, MCF-7 cells treated

with sublethal doxorubicin exhibited increased migration as assessed by collagen matrix assays as well as invasion through matrigels. These phenotypes were prevented by the use of Dasatinib, a Src inhibitor.

5.2 Implications and significance

The studies presented in this thesis have a number of implications for understanding cellular signaling pathways, bioactive sphingolipids and reach into potential applications for cancer therapy.

We establish for the first time nSMase2 as a downstream effector of the DNA damage pathway controlled by ATR, Chk1 and p53. The DNA damage pathway is one of the major pathways involved in tumorgenesis as well as tumor therapy. There is a growing layer of evidence that the DNA damage pathway controls sphingolipids levels and these lipids might be very important downstream effectors that regulate many of the biologies controlled by the DNA damage pathway. From a mechanistic perspective, these studies raise the question of whether nSMase2 is a direct p53 target.

The understanding of the DNA damage pathway is important for targeted manipulations in order to improve chemosensitivity. Many of the clinical trials using inhibitors of DNA damage proteins in cancer therapy failed because of high toxicities of these compounds. This provide a novel potential target of which non-kinase inhibitors can be designed that would be expected to have decreased toxicities.

From a mechanistic point of view, the transient nature of the S phase checkpoint has made it a less attractive checkpoint to study and undermined its importance. As such, the mechanisms regulating the S phase checkpoint remain largely unknown in mammalian cells. This study

implicates, for the first time, a lipid generating enzyme in the establishment of S phase. Furthermore, it provides a potential pathway through activation of a phosphatase and regulating the cohesion complex. This opens questions as to how the phosphatase as well as the cohesion complex are regulating the molecular mechanisms of S phase.

Therapeutically, these studies suggest that nSMase2 would be an attractive therapeutic target to explore for cancer treatment. Recent evidence has suggested that nSMase2 controls invasion and metastasis through exosomes secretion. This study provides an extra layer of evidence for the rational targeting of nSMase2 as it would lead to enhanced chemosensitivity as well as inhibition of invasive phenotype of the cancer cell.

Finally, in the ceramide-centric universe of sphingolipids, this study elaborates on the "many ceramides" hypothesis, the notion that different chain length of ceramides in different compartments can have different biologies. This study perhaps constitute the best evidence yet for this phenomenon as a selective nuclear C18:1 ceramide pool appears to be regulated by DNA damage proteins. As such, targeted manipulation of this pool might constitute a great therapeutic potential that remains to be explored

5.3 Future directions and concluding remarks

This thesis presented novel findings about the role and regulation of nSMase2 in response to DNA damage. While most of these studies have been conclusive, a lot of mechanistic insight remains to be explore. Furthermore, the broad significance of these results in *in vivo* tumor models need to be assessed. In this section, I will address the main points where this project can be developed further

5.3.1 Determining the mechanism of nSMase2 trancriptional induction following doxorubicin

nSMase2 activation in response to Topoisomerase inhibition

The data suggests that nSMase2 induction is very specific to topoisomerase inhibitors. Topoisomerase inhibitors have been described to have multiple mechanisms of action such as reactive oxygen species formation. Our data suggests that the activation of nSMase2 is dependent on the ssDNA damage function of these inhibitors. However, UV, which causes ssDNA breaks as well, does not activate nSMase2 to the same extent. As such, there is the possibility of a DNA damage independent function of topoisomerase inhibitors that is contributing to the induction of nSMase2. To address that, the generation of CRISPR MCF-7 cells that carry one of the mutations in Top1 that confer camptothecin/doxorubicin resistance. These mutations include G365S, G717R and N421R (411). Since these mutations are not dominant negative mutations, there is a need for the use of CRISPR/Cas9 system to introduce these mutations into MCF-7 rather than overexpress the plasmids. Once these generated, their testing for nSMase2 induction would reveal if the top1 inhibition/DNA damage function of camptothecin and doxorubicin is the only function responsible for nSMase2 induction

The hunt for the mechanism of nSMase2 transcriptional induction

One of the major points that this thesis does not address is the precise mechanism of nSMase2 upregulation downstream of p53, and whether nSMase2 is a direct p53 target. Many studies in the literature have attempted to identify direct p53 targets through different methods. However, none of these presented nSMase2 as one of the genes identified (412,413). This could be explained by the possibility that these studies were looking at the incorrect isoform of nSMase2

and possibly trying to map p53 binding sites upstream of exon1. The data from this thesis suggest that there is a novel, previously undescribed isoform of nSMase2 with a novel TSS between exon 2 and exon3. As such, there is a need to characterize the complete new transcript and map the 5'UTR region of nSMase2 upstream of exon 3. The characterization of the 5'UTR can be done through further optimization of the RACE protocol as well as through SMART cell sequencing technology. Once the 5'UTR is identified, a characterization of the alternative promoter responsible for this novel TSS would be characterized. The first 2000 bp upstream of the newly identified TSS should be cloned and tested for promoter activity. Furthermore, this can be helped through the use of promoter predicting softwares such as genomatix. Interestingly, this promoter would most likely be a TATA-less promoter as analysis of the 21kb region in intron2 did not reveal any TATA boxes. Following that, the characterization of the minimal promoter element necessary for nSMase2 induction will allow prediction of possible transcription factors responsible for this induction. Validation of these by mutations of the promoter sequences as well as knockdown of transcription factors could be made for verification. Furthermore, the relation of the identified transcription factor to p53 could be explored if it was not described previously in the literature

The possible involvement of delta p53

The involvement of p53 in the establishment of S phase checkpoints is subject to debate in the literature. The main points of discussion is whether p53 is necessary or not for establishment of S phase checkpoint. The general consensus has long been that p53 can signal in S phase but is not required for the establishment of the S phase checkpoint (414). However, a breakthrough was made when deltap53, a shorter form of p53 was found to be selectively induced during S phase in an ATR-dependent manner (392). This form of p53 induces p21 to selectively repress transcription of S phase genes such as cyclins and Cdks and promote S phase arrest. This was found to be an

alternative mechanism of S phase establishment (392). In this context, delta p53 might realistically be the isoform that is mediating nSMase2 induction. This could account for the discrepancy seen with the p53 effects on nSMase2 following doxorubicin and following overexpression of WT p53. As such, experiments that assess the role of delta p53 in nSMase2 induction would be interesting to carry out. This would include cloning delta p53 construct and overexpressing it in comparison with WT p53. Ideally, this should result in a more robust nSMase2 induction. Furthermore, assessing whether nSMase2 activation falls downstream of p21 would be necessary. This can be carried out through siRNA knockdown of p21 and testing for nSMase2 induction.

5.3.2 Characterizing nSMase2 localization to the nucleus

The finding that nSMase2 localizes to nuclear speckles is one that raises many questions not only from an enzymatic point of view, but also from the lipids perspective. One of the main questions that remain unresolved is how nSMase2 localizes to the nucleus. This can be divided further into two layers of questions. The first layer pertains to whether this is a translocation or a localization of a *de novo* synthesized enzyme. Our data so far suggests it is the latter but we do not have conclusive evidence of that, partly because nSMase2 exists in very low amounts in MCF-7 endogenously. As such, it is very hard to monitor its localization without stimulating its levels and as ac consequence we do not possess evidence of its baseline localization. The second layer of questions pertains to how nSMase2 localizes to the nucleus. We did try to address this issue unsuccessfully. In general, an enzyme can localize to the nucleus through three main mechanisms: the first requires it to possess a nuclear localization signal (NLS), the second is by passive diffusion (if the protein is smaller than 30 kDA), and the third is through intereaction with a protein that translocates to the nucleus. Of these 3 mechanisms, we think the third is the most probable. However, we do not have evidence of it yet. As a matter of fact, we know that nSMase2 has a size

of 71 kDA and thus can't passively diffuse through nuclear pores. The analysis of nSMase2 amino acid sequence revealed a putative NLS composed of the positive charged residues lysine and arginine. Cloning of that putative NLS into a GFP vector did not increase the localization of GFP to the nucleus suggesting that it is not sufficient to induce translocation of nSMase2. These results leave protein-protein interaction as the only remaining possibility. To probe that, we tried co-expression of WT nSMase2 with EED, the only known protein that directly interacts with nSMase2. We did observe that this interaction does not occur under basal conditions and under conditions of treatment with doxorubicin. It is very possible that a yet unidentified interaction partner is responsible for nSMase2 translocation to the nucleus. One experiment that would attempt to solve that question is to try and pull down endogenous nSMase2 and send it to mass spectrometry to identify potential interaction partners following doxorubicin treatment. Of these, the nuclear proteins identified can be tested for their ability to transport overexpressed WT nSMase2 to the nucleus. Another hypothesis is that the unidentified 5'UTR of nSMase2 upstream of exon3 is regulating its localization. It is not unheard of that alternative forms of a certain enzyme have different localization. However, this would be very novel as that would be the first case where a 5'UTR would be regulating the nuclear localization of a protein. As such, the experiment to do would be to clone the full hnRNA sequence into a vector and overexpress and see if that changes the localization of nSMase2.

Another layer of questions comes in the form of nuclear speckles. It is interesting that nSMase2 appears to localize to these nuclear structures containing phospholipids suggesting that these are hydrophobic enough to contain lipids. However, numerous questions arise about the structure and the function of thee nuclear speckles. From a structural perspective, it is unknown whether these speckles are membrane-bound nuclear structures and if so whether their membranes

are monolayers or bilayers. Functionally, questions arise about whether these lipids are mediating some of the functions of these nuclear speckles such as RNA splicing and regulation of splice factors, and if so how.

There are also questions on how nSMase2 gets activated in these nuclear speckles. NSMase2 has been shown to be activated by anionic phospholipids such as PA and PS, however, given the abundance of phospholipids in the nuclear speckles, is it possible that nuclear nSMase2 has a different activation mechanism than plasma membrane bound nSMase2? Do PIPs activate nSMase2? And if so, are there any specificity in which PIP activate nSMase2? These questions remain unanswered. One possible experiment to perform to start exploring this is to check if PIPs activate nSMase2 *in vitro*. This would be done using different PIP species to see if there is specificities for one over the other.

5.3.3 Reconciling results of the yeast studies on Isc1 and mammalian studies on nSMase2: possibilities for an evolutionary role of the enzyme

Work from our lab has suggested that the yeast orthologue of nSMase2 has similar protective functions to nSMase2. Isc1 plays a protective role in response to Hydroxyurea. This happens through the regulation of the spindle checkpoint by preventing collapse of microtubules. Downstream effectors of Isc1 in that biology include CDC55 and Swe1. Interestingly, the lipid identified to mediate Hydroxyurea sensitivity is C18:1 phytoceramide. Recent unpublished work also point to Isc1 nuclear localization. All of these data suggest a very similar conserved evolutionary function between nSMase2 and Isc1 (Table 2). Moreover, phosphoproteomics data done on Isc1 revealed similar changes in cohesion condensing protein complexes. One big difference however is that Isc1 regulation appears to be post-transcriptional and involves mediators

of the spindle checkpoint such as Bub1 and Mad1. In contrast nSMase2 appears to be regulated transcriptionally in the DNA damage response. One set of questions that arise from this comparison is whether nSMase2 has any function in the mammalian spindle checkpoint. To study that, one could look at drugs that stimulate the spindle checkpoint such as the taxanes (taxol being an example) or the vinca alkaloids (vinblastine or vincristine). The expected finding is that one of these should upregulate nSMase2, either transcriptionally or post-transcriptionally, to mediate similar effects than the one seen with doxorubicin.

5.3.4 Determining if nSMase2 inhibition is a viable clinical avenue to consider by performing animal models studies

The findings from this thesis suggest that nSMase2 inhibition might be beneficial in the context of chemotherapy. In order to assess that fully, mouse models of breast cancer should be used. Both xenografts and genetic would be good models to use. From the xenograft perspective, the use of MCF-7 cells that possess CRISPR knockout of nSMase2 genes would be a good start point. The idea is to compare xenografts with and without nSMase2 for response to anthracycline chemotherapy in breast cancer. Genetic models can also be used, such as the MMTV/Wnt genetic model of breast cancer. In that context, the use of GW4869, an nSMase2 inhibitor would be beneficial. The idea would be to evaluate chemotherapy versus chemotherapy with GW4869 for effectiveness in reducing tumor size and outcomes in mice. One of the drawbacks of that is that GW4869 has very low solubility and is difficult to use. A way around this would be to cross the MMTV/Wnt mice with the fro/fro mice that possess a catalytically inactive nSMase2. The resulting mice should be compared to the MMTV/Wnt mice in its response to anthracycline chemotherapy

Figures

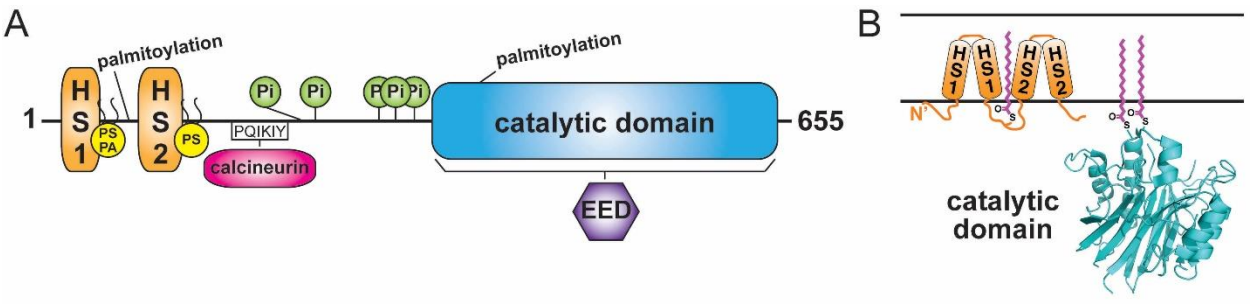


Figure 1: Domain architecture and membrane topology of nSMase2

(**A**) Domain architecture of nSMase2. Catalytic domain, blue; Hydrophobic segments 1 (HS1) and 2 (HS2), orange; Anionic phospholipid binding sites, yellow; Phosphorylations sites (Pi), green; Calcineurin binding motif, magenta; EED binding region, purple; and palmitoylation sites are indicated. (**B**) Membrane topology of nSMase2 with palmitoylation sites, magenta. HS1 and HS2 associate with, but do not transverse, the membrane. A representative structure of the nSMase2 catalytic domain is shown from *B. Cereus* sphingomyelinase (PDB ID 2DDT).

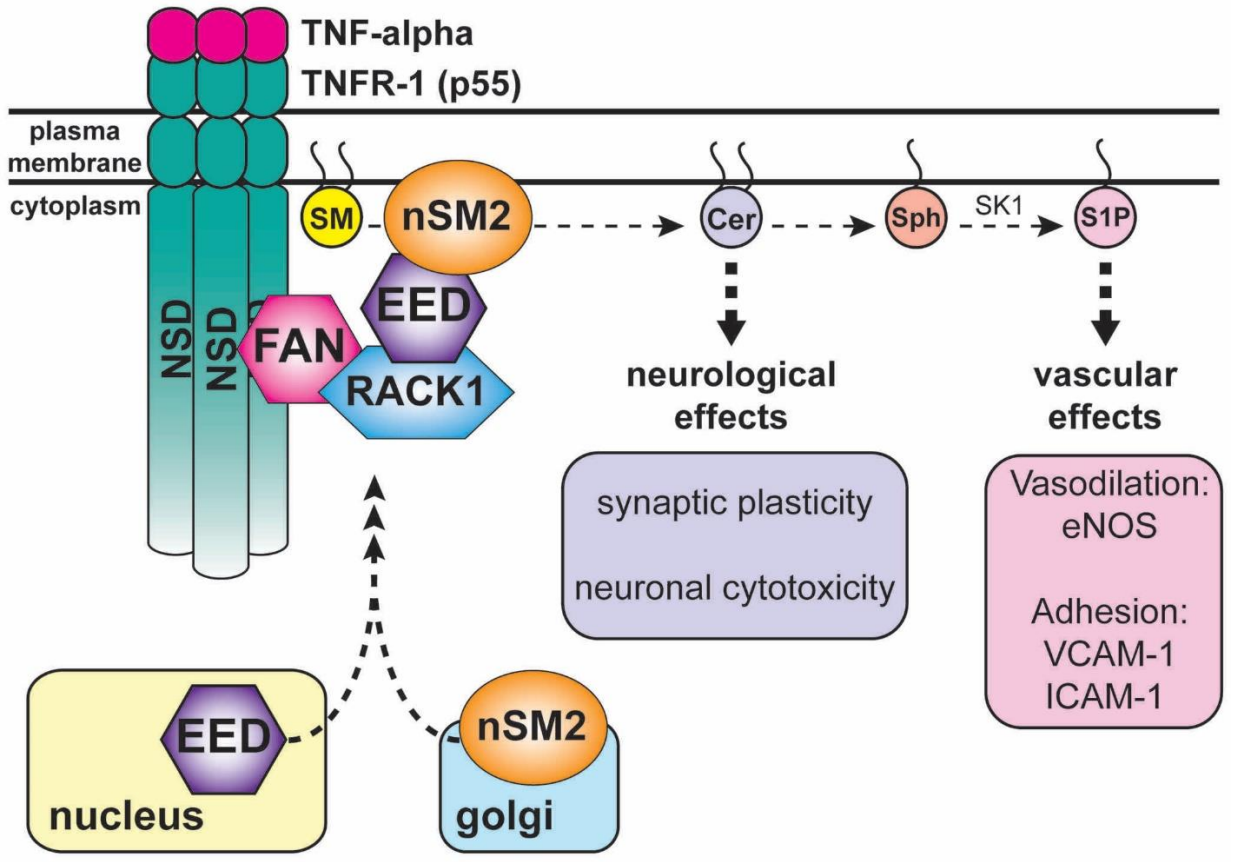


Figure 2: nSMase2 is a mediator of TNF-α signaling

TNF-α induces nSMase2 activation and ceramide generation. Recruitment of nSMase2 to the plasma membrane occurs through formation of a complex between five proteins: nSMase2, EED, RACK1, FAN, and TNFR-1.

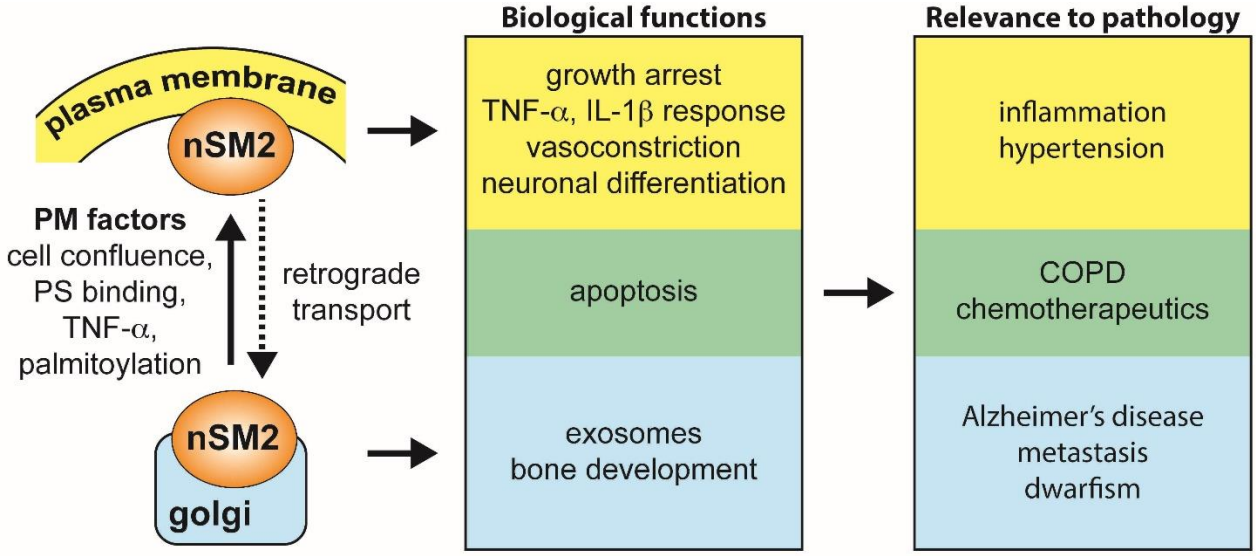


Figure 3: Summary of nSMase2 functions and relevance to pathology

Association of nSMase2 function in different cellular compartments. Plasm membrane, yellow; Golgi, blue; Unknown, green.

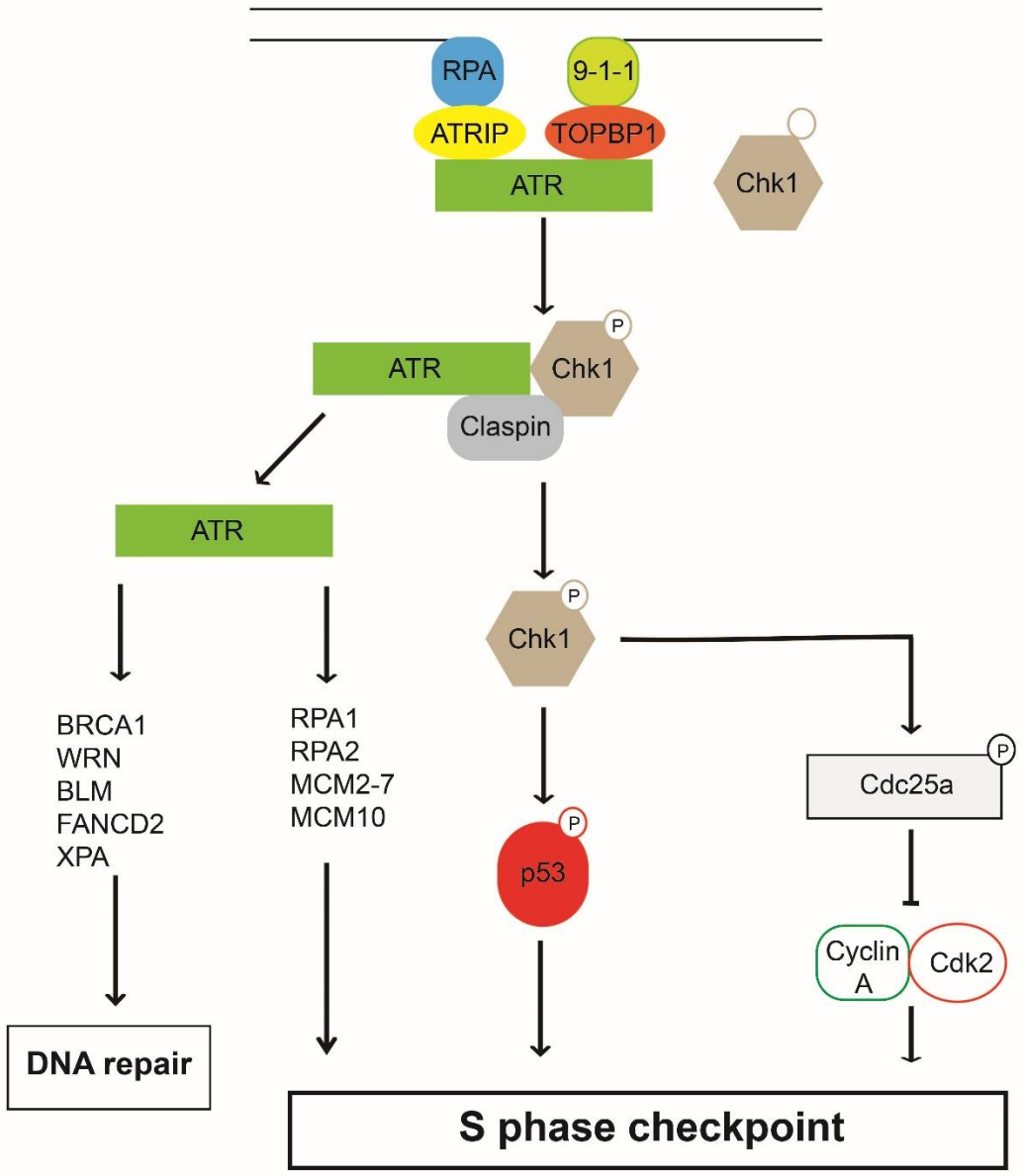


Figure 4: Regulation of the S phase checkpoint

Scheme showing the central role of ATR and Chk1 in controlling the S phase checkpoint

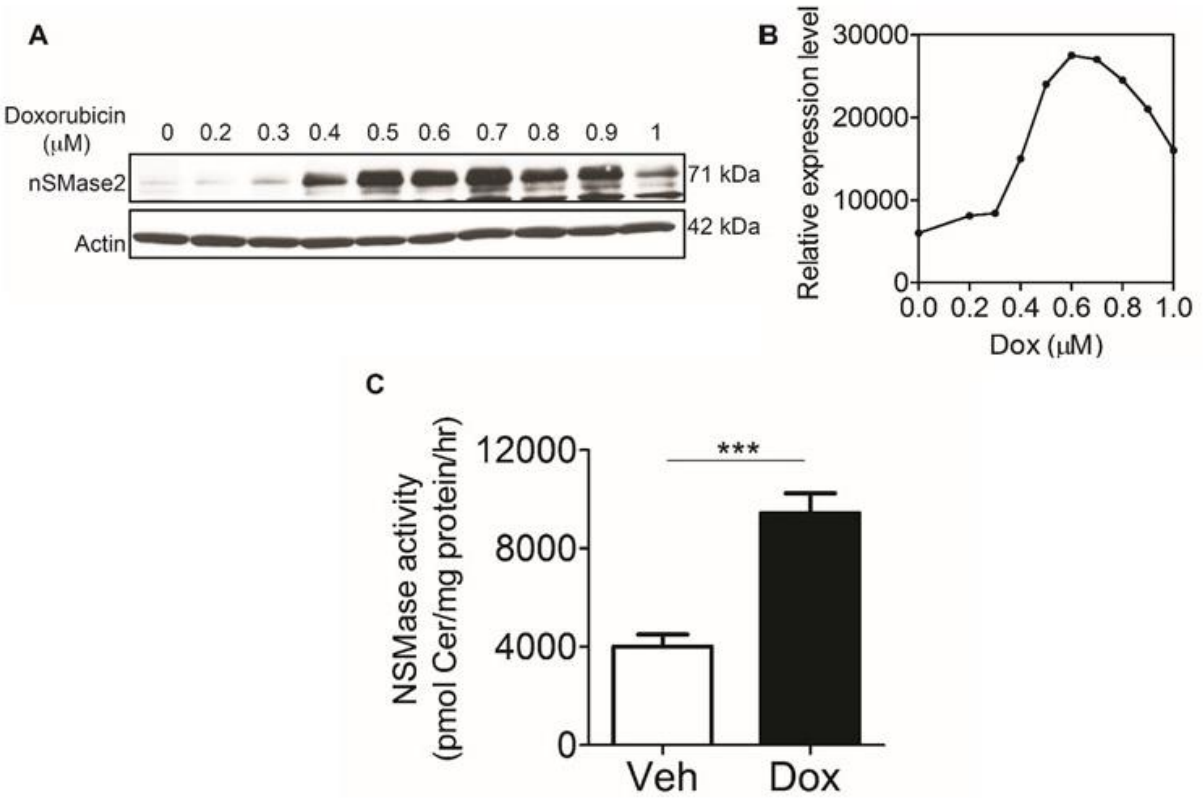


Figure 5 A-C: Doxorubicin induces nSMase2 upregulation and ceramide increase in MCF-7 cells

(**A**) MCF-7 cells were plated in 60 mm dishes and treated with either DMSO or Doxorubicin at different doses for 24 hours. Cells were collected, lysed and immunoblotted as described under "Experimental Procedures". (**B**) Quantification of nSMase2 induction by ImageJ and normalization to actin. (**C**) MCF-7 cells were plated in 60 mm dishes and treated for 24 hours with either DMSO (Veh) or doxorubicin (Dox). Cells were collected and *in vitro* NSMase assay was performed as described under "Experimental Procedures", ***$p<0.001$

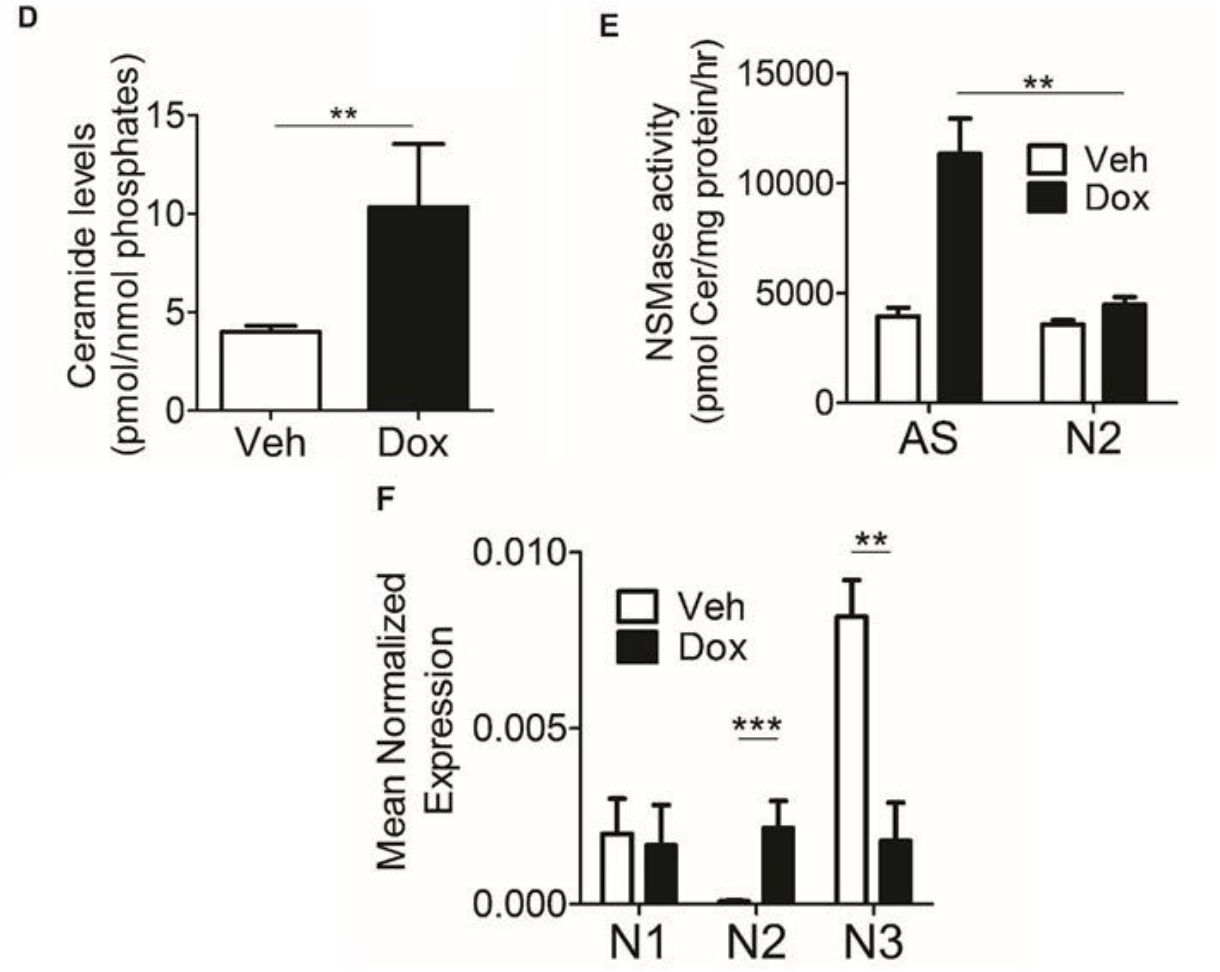


Figure 5 D-F: Doxorubicin induces nSMase2 upregulation and ceramide increase in MCF-7 cells

(**D**) MCF-7 cells were collected 24 hours after treatment and analyzed for sphingolipids by LC/MS mass spectrometry, **p<0.01. (**E**) MCF cells were plated in 60 mm dishes. 24 hours later, siRNA to All Star negative control (AS) or nSMase2 (N2) were transfected for 24 hours after which either vehicle or doxorubicin were added. Cells were collected after 24 hours, lysed and *in vitro* sphingomyelinase assay was performed, **<p<0.01 (**F**) MCF-7 cells were lysed 24 hours after treatment. RNA was isolated and transformed to cDNA to be quantified by qRT-PCR using primers for neutral sphingomyelinase-1 (N1), nSMase2 (N2) and neutral sphingomyelinase-3 (N3) as described, **p<0.01, ***p<0.001.

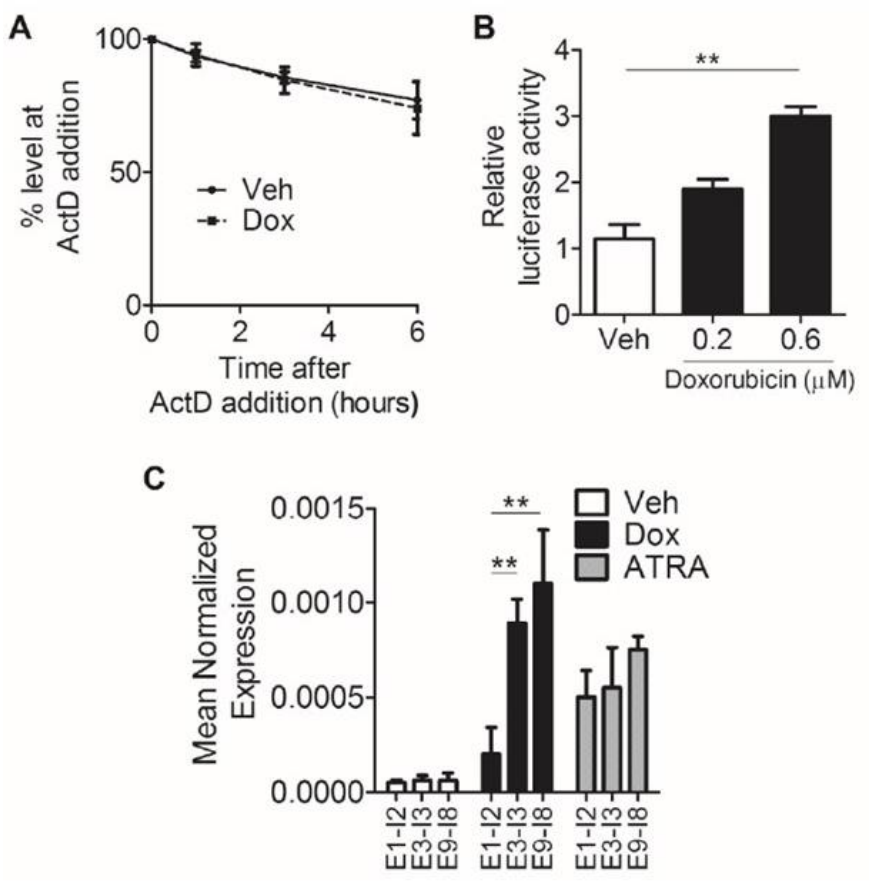


Figure 6: nSMase2 is upregulated transcriptionally via a novel TSS

(**A**) MCF-7 cells were seeded in 60 mm dishes and treated with Vehicle or Doxorubicin for 24 hours. After that, 10 nM of actinomycin D was added for 1, 3 and 6 hours. Cells were lysed, RNA was isolated and transformed to cDNA and qRT-PCR was performed with nSMase2 primers. (**B**) MCF-7 cells were seeded in 60 mm dishes and transfected with LacZ and pGL3 basic vector containing the first 1600 bp upstream of exon1 for 24 hours after which cells were treated with doxorubicin. Cells were then collected and both luciferase and β galactosidase assays were performed as described under "Experimental Procedures", **p<0.01. (**C**) Cells were treated with Vehicle, Doxorubicin for 24 hours after which cells were collected, RNA was isolated and transformed to cDNA, qRT-PCR was performed using exonic-intronic primers, exon1-intron2 (E1-I2), exon3-intron3 (E3-I3) and exon9-intron8 (E9-I8), **p<0.01, ***p< 0.001.

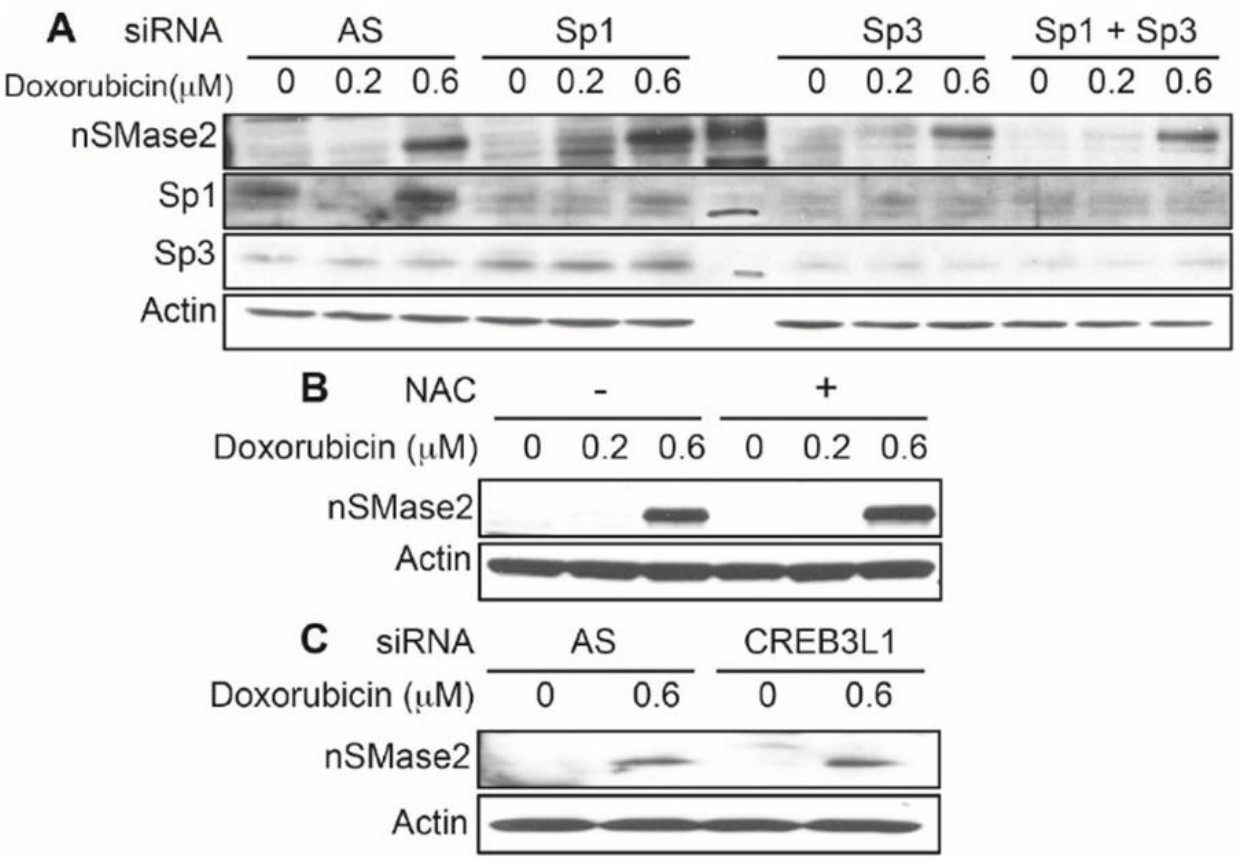


Figure 7: nSMase2 upregulation is independent of known transcriptional regulators

(**A**) MCF-7 cells were seeded in 60 mm dishes and transfected with siRNA to All Star negative control (AS), Sp1, Sp3 or both together. 24 hours later cells were treated with Vehicle, 0.2 μM or 0.6 μM doxorubicin. After 24 hours, cells were collected and immunoblotted for nSMase2, Sp1, Sp3 and Actin. (**B**) MCF-7 cells were seeded in 60 mm dishes. 1 hour before stimulation with doxorubicin or vehicle, they were pre-treated with N-acetylcysteine (NAC). Cells were collected and immunoblotted for nSMase2 and actin. (**C**) MCF-7 cells were seeded in 60 mm dishes and transfected with siRNA to AS or CREB3L1. 24 hours later, they were treated with vehicle or 0.6 μM doxorubicin. Cells were collected and immunoblotted for nSMase2 and actin.

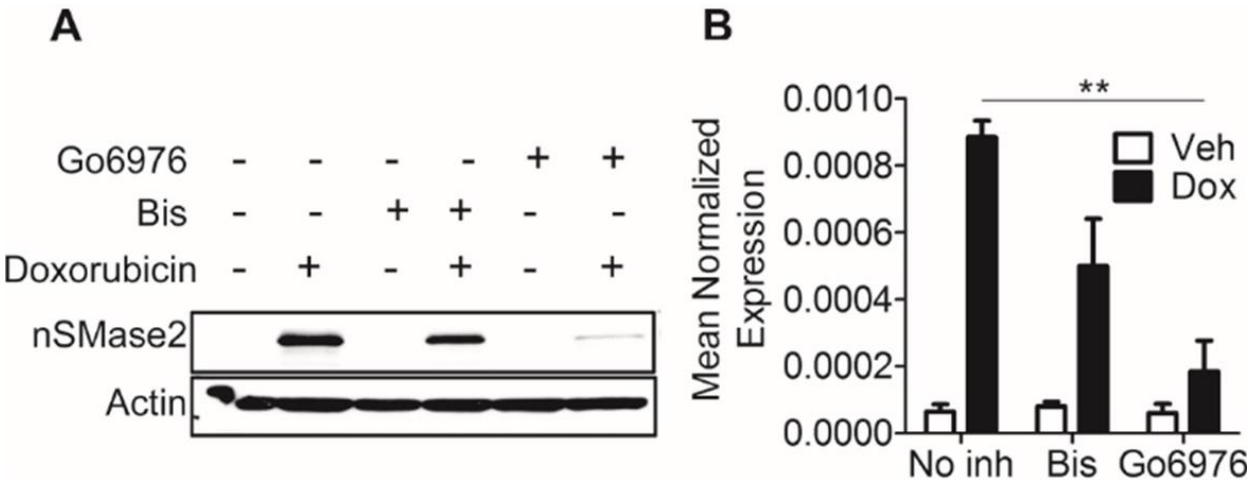


Figure 8 A-B: G06976 regulates nSMase2 transcriptionally

(**A-B**) MCF-7 cells were seeded in 60 mm dishes and, 1 hour prior to stimulation with either vehicle or doxorubicin, were pre-treated with the specified inhibitor and 24 hours after treatments, cells were collected and immunoblotted for nSMase2 and actin (A), or RNA was isolated and transformed to cDNA and qRT-PCR was performed for nSMase2 (B), **$p<0.01$

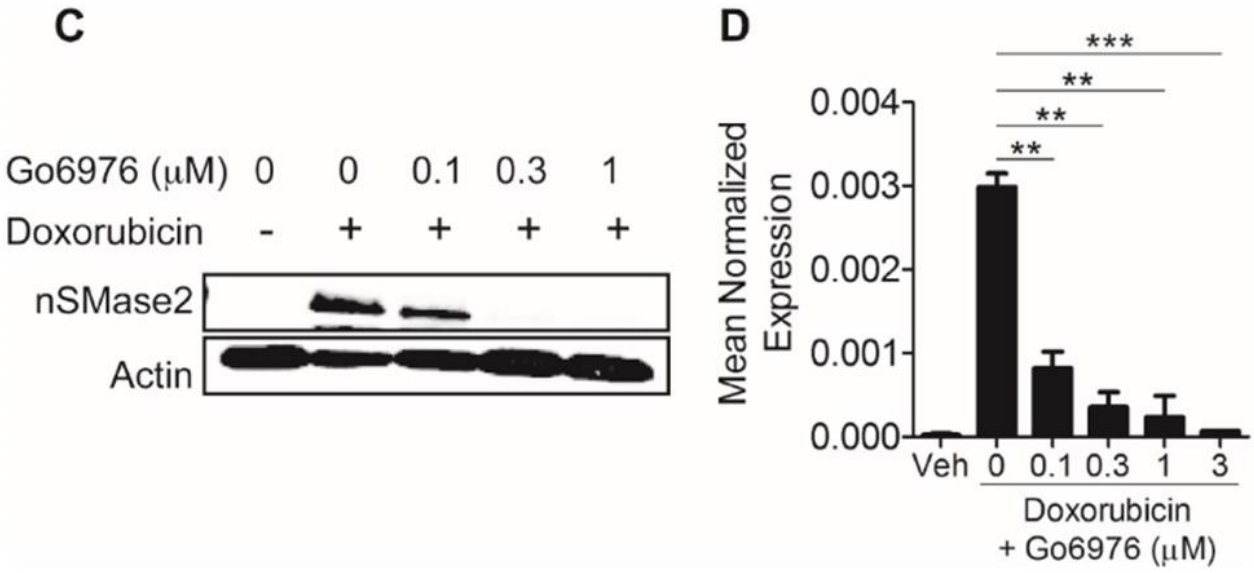


Figure 8 C-D: G06976 regulates nSMase2 transcriptionally

(**C-D**) MCF-7 cells were seeded in 60 mm dishes and, 1 hour prior to stimulation with either vehicle or doxorubicin, were pre-treated with the specified inhibitor and 24 hours after treatments, cells were collected and immunoblotted for nSMase2 and actin (C), or RNA was isolated and transformed to cDNA and qRT-PCR was performed for nSMase2 (D), **p<0.01, ***p<0.001.

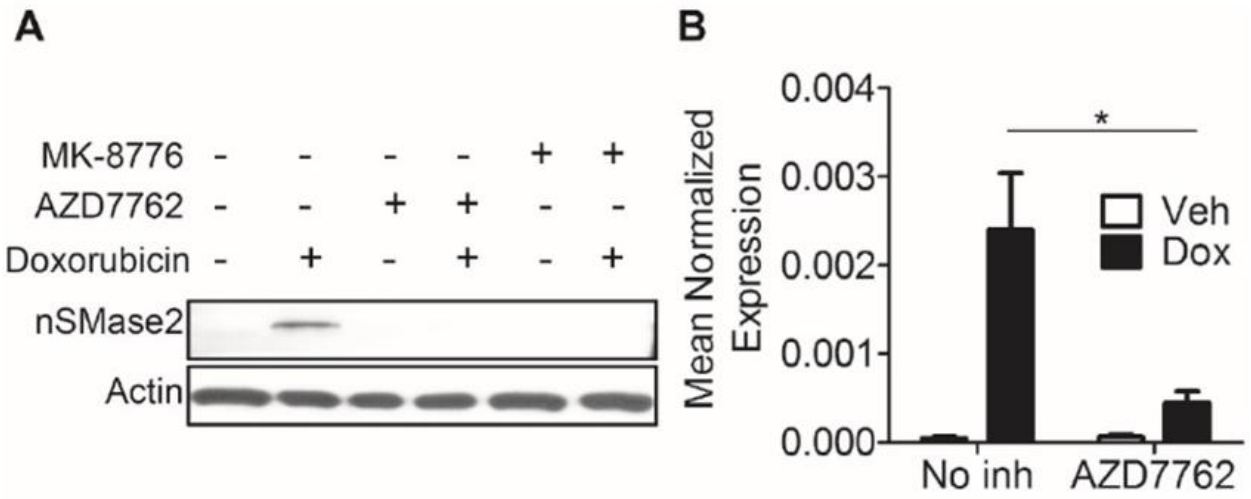


Figure 9 A-B: Chk1 regulates nSMase2 transcriptionally

(**A-B**) MCF-7 cells were seeded in 60 mm dishes and were pre-treated with the specified inhibitor 1 hour prior to stimulation with either vehicle or doxorubicin. 24 hours after treatment, cells were collected and immunoblotted for nSMase2 and actin (A), or RNA was isolated and transformed to cDNA and qRT-PCR was performed for nSMase2 (B). *$p<0.05$

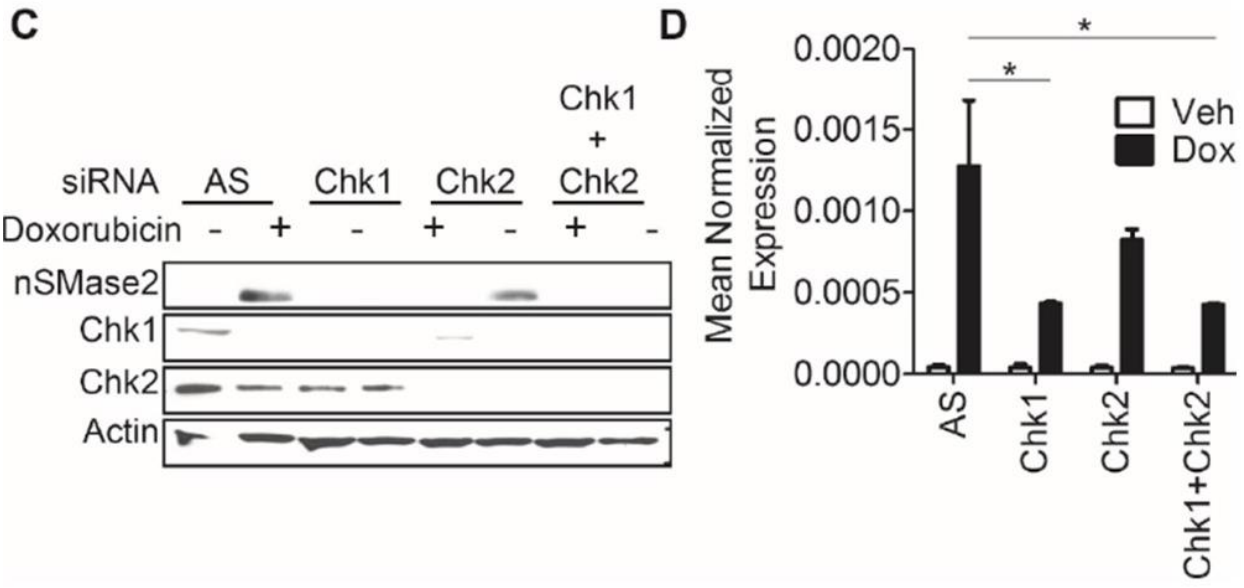


Figure 9 C-D: Chk1 regulates nSMase2 transcriptionally

(**C-D**) MCF-7 cells were seeded in 60 mm dishes and siRNA was performed using All Star negative control (AS), Chk1, Chk2 or Chk1 + Chk2. 24 hours later, cells were stimulated with either vehicle or doxorubicin and collected for analysis by immunoblotting (C) or qRT-PCR (D), *$p<0.05$.

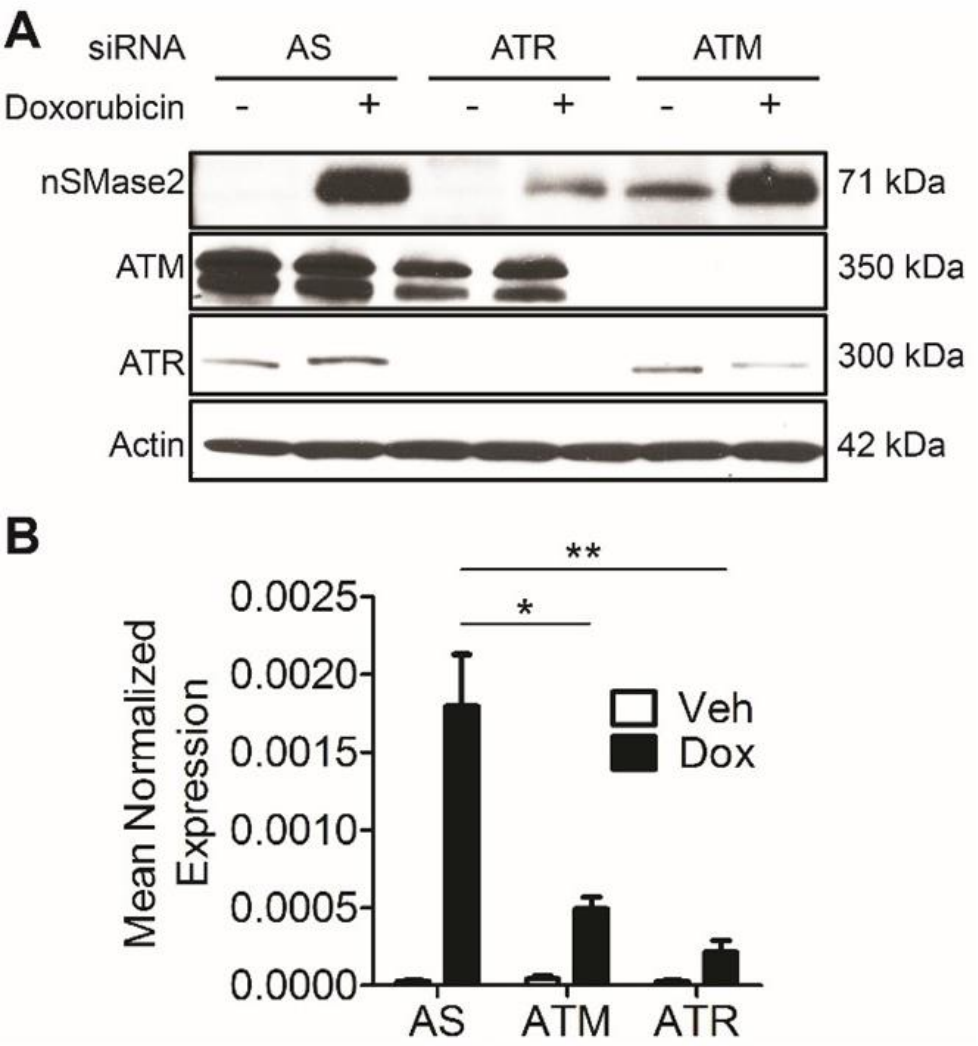


Figure 10: ATR regulates nSMase2 transcriptionally upstream of Chk1

(**A-B**) MCF-7 cells were seeded in 60 mm dishes, and siRNA knockdown was performed using AS, ATM or ATR for 24 hours. After that, vehicle or doxorubicin were added and cells were collected and imuunoblotted for nSMase2, ATM, ATR and actin (A), or RNA was isolated and transformed to cDNA and qRT-PCR was performed for nSMase2 (B), *$p<0.05$. **$p<0.01$.

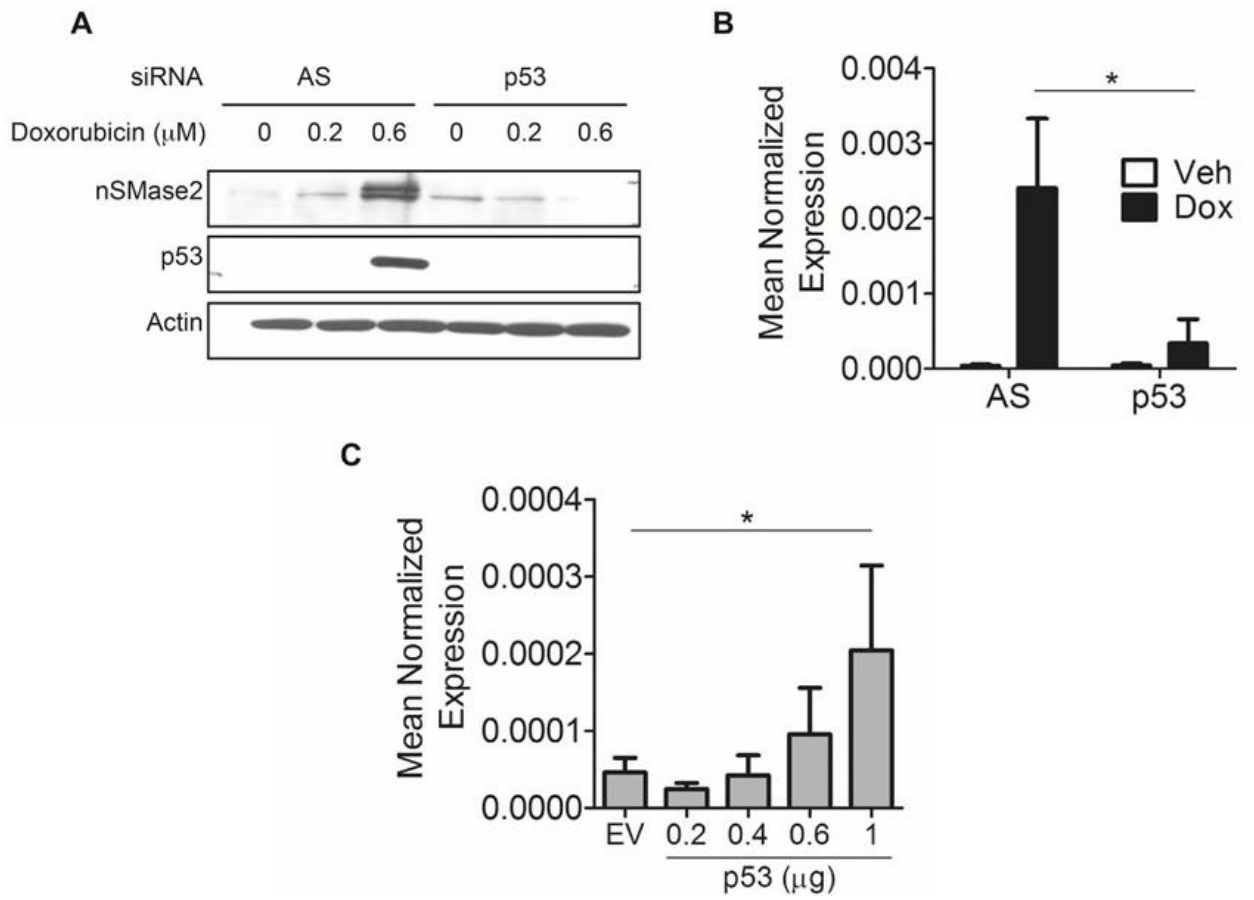


Figure 11 A-C: p53 is both necessary and sufficient for the induction of nSMase2

(**A-B**) MCF-7 cells were seeded in 60 mm dishes and siRNA knockdown was performed using AS or p53 for 24 hours. After that, vehicle or doxorubicin were added and cells were collected and immunoblotted for nSMase2, p53 and actin (A), or RNA was isolated and transformed to cDNA and qRT-PCR was performed for nSMase2 (B), *$p<0.05$. (C) MCF-7 cells were seeded in 60 mm dishes and transfected with either control (pcdna) or increasing concentrations of wild-type p53 plasmid, * $p<0.05$.

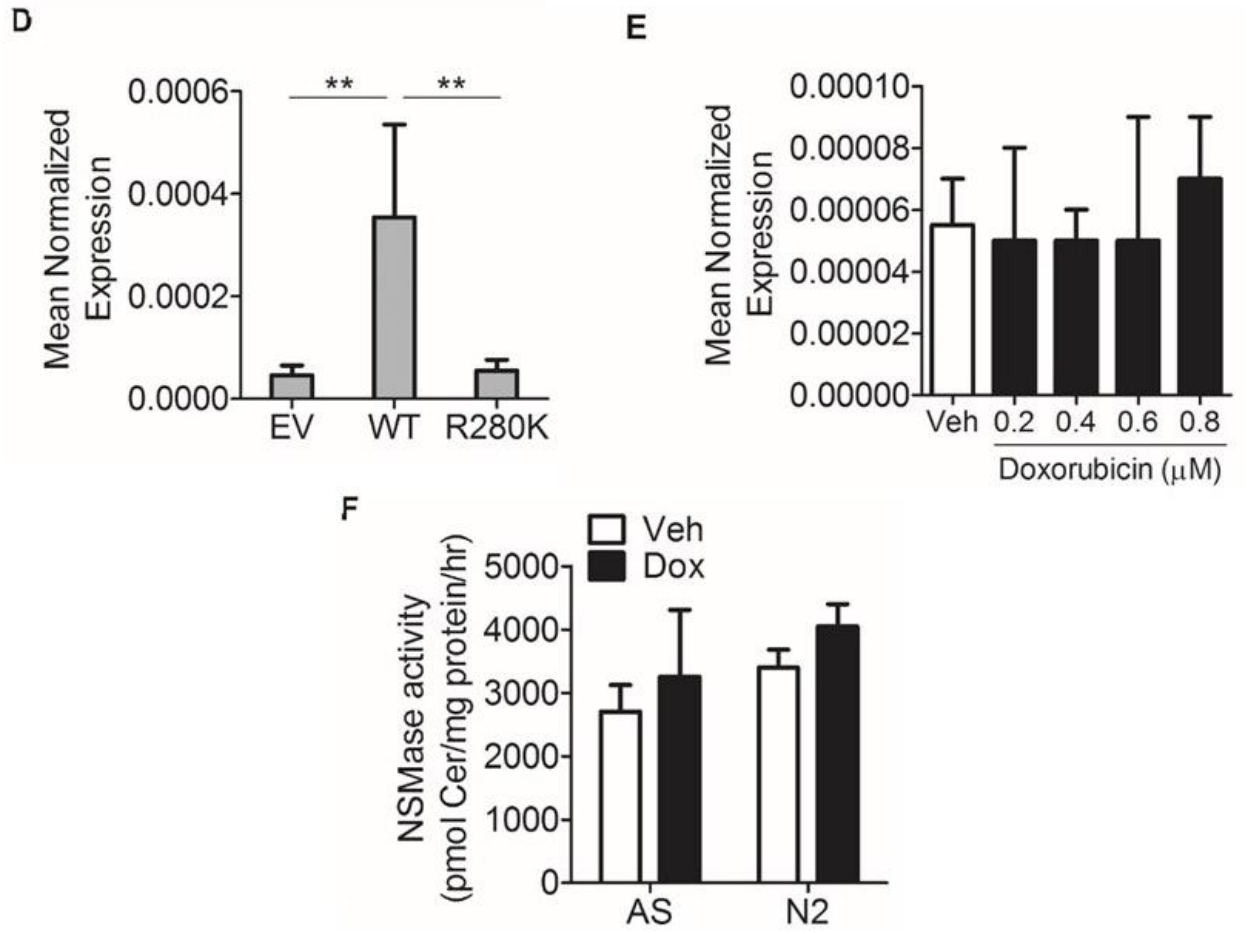


Figure 11 D-F: p53 is both necessary and sufficient for the induction of nSMase2

(**D**) MCF-7 cells were seeded in 60 mm dishes and transfected with either control (pcdna) or different p53 mutants, ** $p<0.01$. (**E**) MDA-MB-231 cells were seeded in 60 mm dishes and treated with increasing doses of doxorubicin. Cells were collected 24 hours later, RNA isolated and transformed to cDNA and qRT-PCR run for nSMase2. (**F**) MDA-MB-231 cells were seeded in 60 mm dishes, 24 hours later siRNA to AS or nSMase2 was transfected. After 24 hours, doxorubicin was added and cells were lysed 24 hours later and *in vitro* neutral sphingomyelinase activity assay was performed as described in "Experimental procedures".

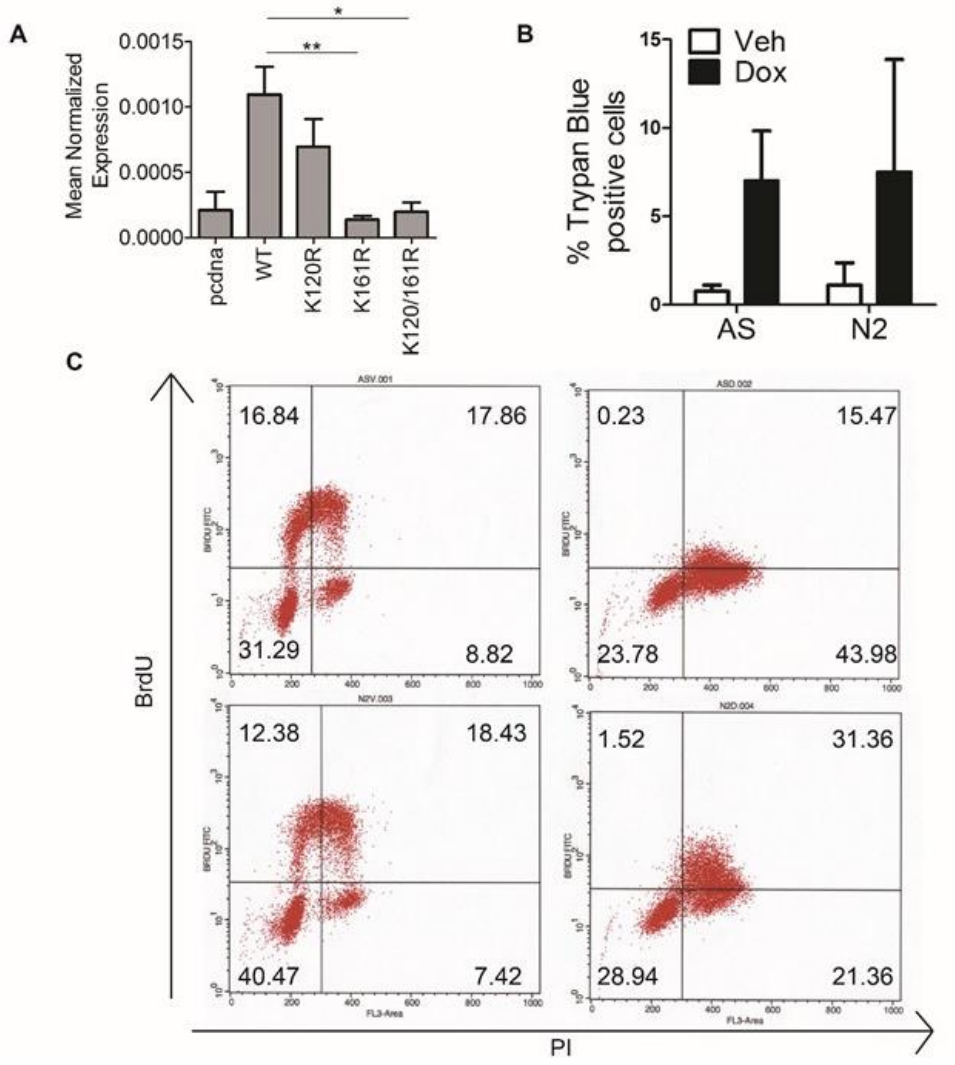


Figure 12: nSMase2 mediates growth arrest in response to doxorubicin

(**A**) MCF-7 cells were seeded in 60 mm dishes and transfected either with control (pcdna) or p53 acetylation mutants, *p<0.05, **p<0.01. (**B**) MCF-7 cells were seeded in 60 mm dishes and siRNA knockdown was performed using AS or nSMase2 for 24 hours. After that, vehicle or doxorubicin were added and cells were collected, stained with Trypan Blue and counted. (**C**) MCF-7 cells were seeded in 60 mm dishes and siRNA knockdown was performed using AS or nSMAse2 for 22 hours. A pulse of BrdU was given to cells for 30 mins and they were collected and analyzed by flow cytometry.

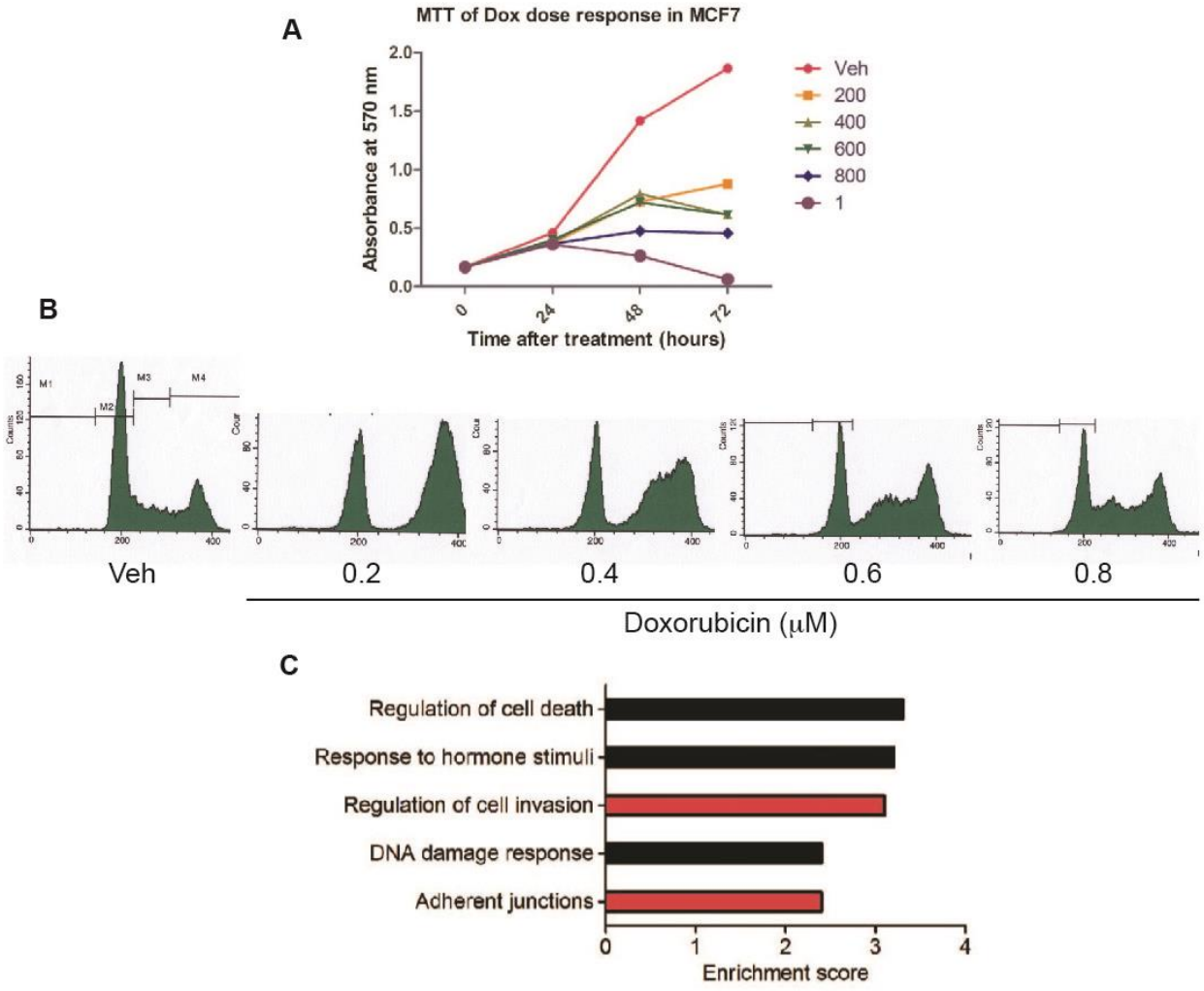


Figure 13: Doxorubicin induces distinct biologies at different doses

(**A**). MCF-7 cells were plated in 6 well plates and treated with different concentrations of doxorubicin for 24 hours after which MTT assays for cell viability were performed according to "Experimental procedures". (**B**) MCF-7 cells were seeded in 60 mm dishes and treated with different concentrations for 24 hours after which they were prepped for flow cytometry as described (**C**) MCF-7 cells were plated in 60 mm dishes and treated with vehicle or 0.6 μM doxorubicin for 24 hours. RNA was then isolated and sent for RNA sequencing as described under "Experimental procedures". Data were plotted on DAVID software and enrichment scores were calculated for the genes upregulated by 2 fold or more. Shown in red are invasive pathways.

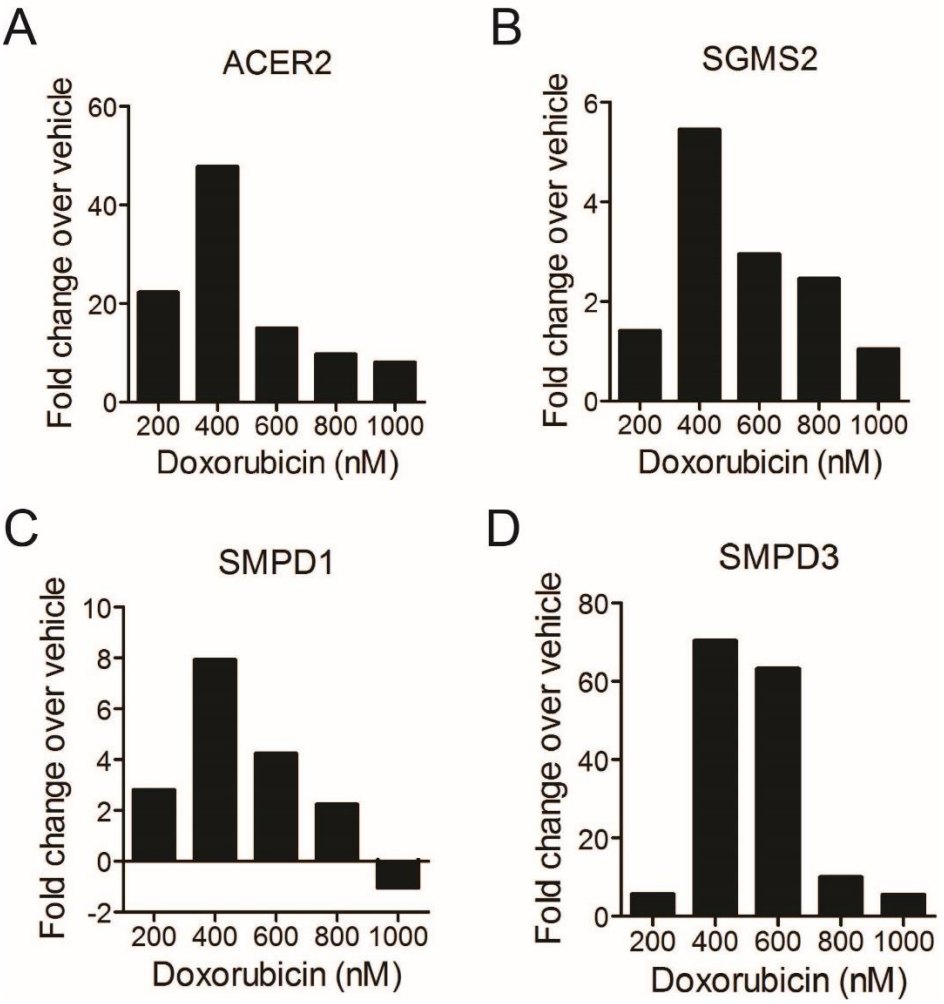


Figure 14: Enzymes with changes in mRNA at low doses of doxorubicin

(**A-D**) MCF-7 cells were seeded in 60 mm dishes and treated with different doses of doxorubicin. After 24 hours, cells were collected and RNA was extracted and transformed to cDNA. The cDNA was run on a qRT-PCR sphingolipid enzymes array as described in experimental procedures.

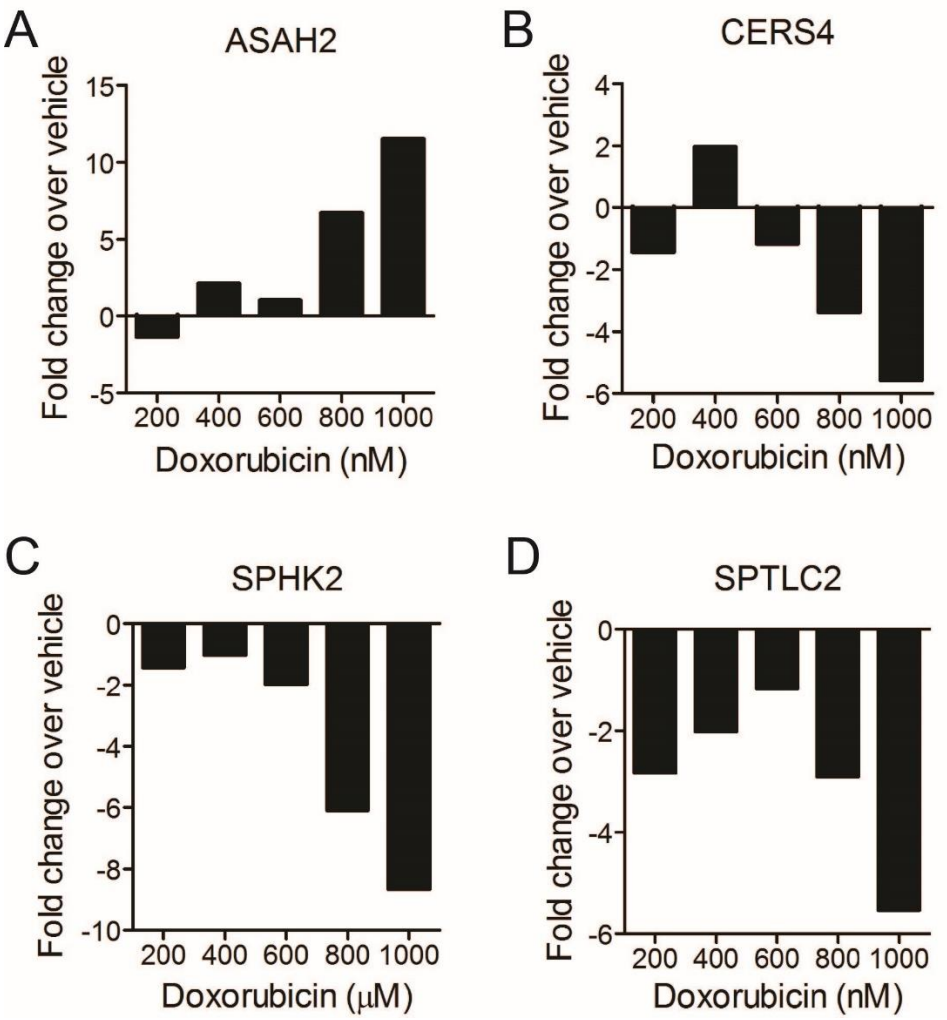


Figure 15: Enzymes with changes in mRNA at high doses of doxorubicin

(**A-D**) MCF-7 cells were seeded in 60 mm dishes and treated with different doses of doxorubicin. After 24 hours, cells were collected and RNA was extracted and transformed to cDNA. The cDNA was run on a qRT-PCR sphingolipid enzymes array as described in experimental procedures.

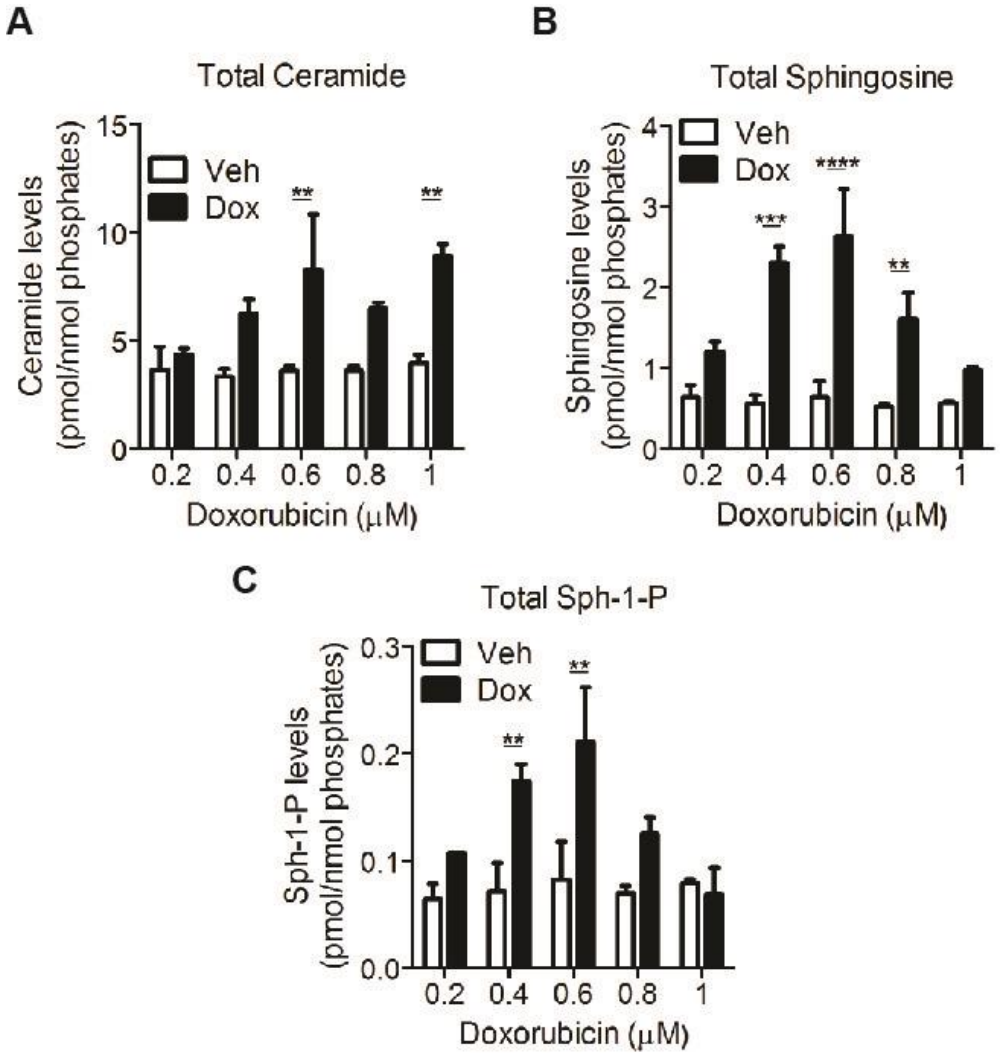


Figure 16: Lipid profile following doxorubicin mirrors enzymatic changes

(**A-C**) MCF-7 cells were seeded in 100 mm dishes and treated with different doses of doxorubicin. 24 hours later cells were harvested in cell extraction medium and sent for the lipid mass spectroscopy core for LC/MS according to experimental procedures.

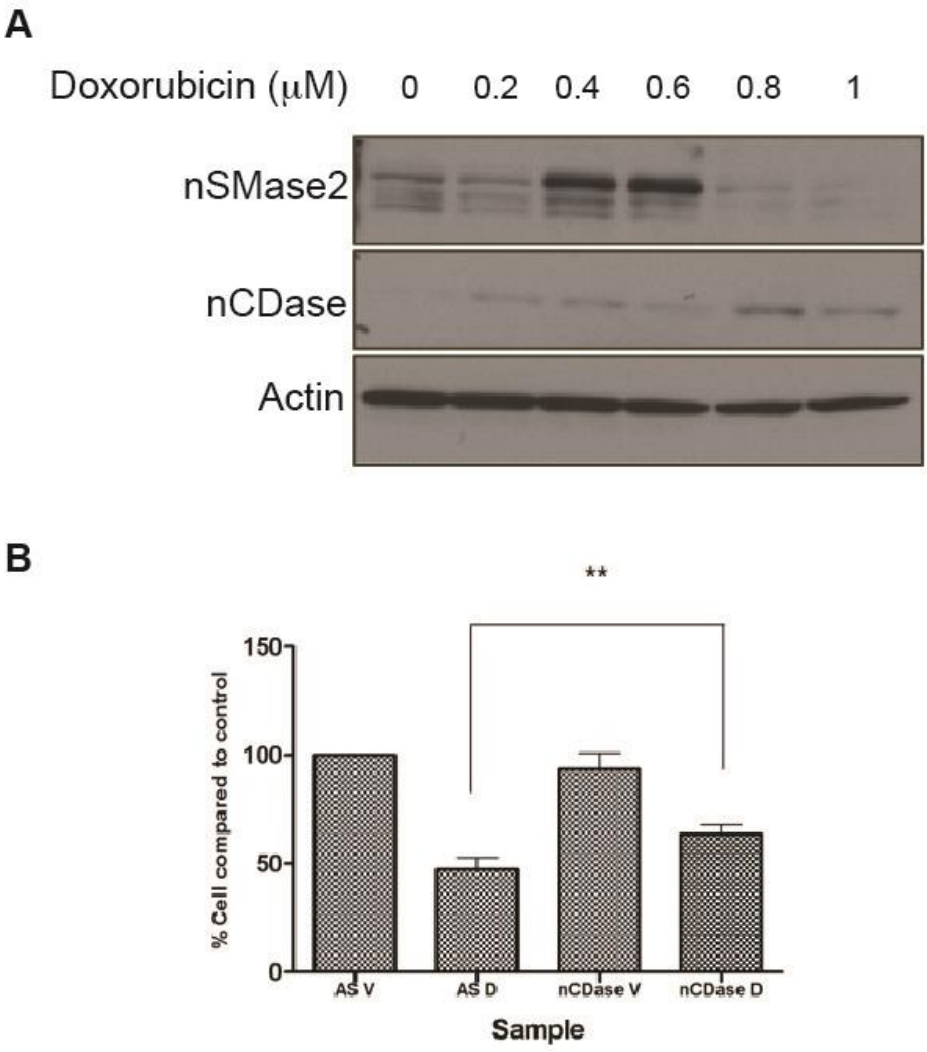


Figure 17: Neutral ceramidase: a potential p53 target involved in cell death

(**A**) MCF-7 cells were plated in 60 mm dishes and treated with different doses of doxorubicin for 24 hours after which they were lysed and immunoblotted for neutral ceramidase (nCDase), nSMase2 and actin. (**B**) MCF-7 cells were seeded in 60 mm dishes and siRNA to All star negative control (AS) or nCDase was transfected. Cells were then treated with either vehicle (V) or 1 μM of doxorubicin for 24 hous and cells were counted using trypan blue; **p<0.01.

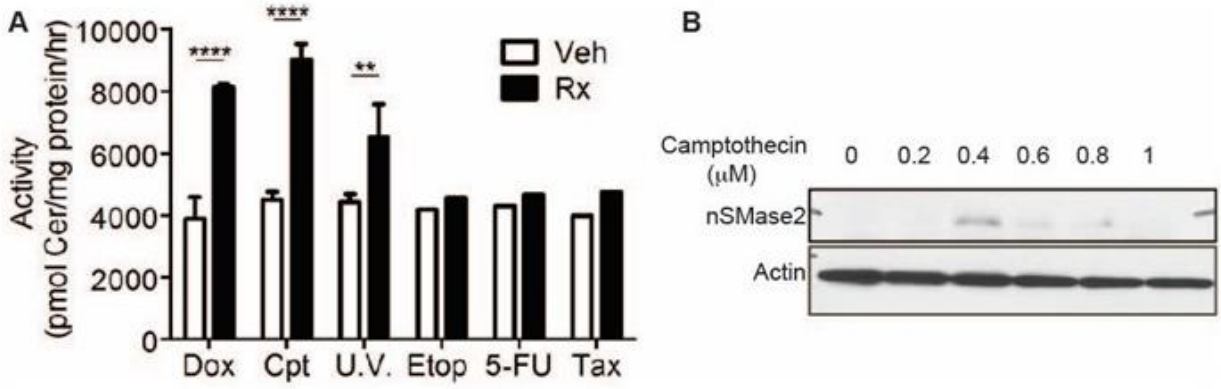


Figure 18 A-B: nSMase2 upregulation in response to DNA damage is specific to ssDNA breaks generating agents

(**A**) MCF-7 cells were plated in 60 mm dishes and treated with vehicle or 0.6 μM doxorubicin, 0.8 μM camptothecin, 10 mJ/cm2 UV, 50 μM etoposide, 10 μM 5-FU or 1 μM taxol for 24 hours after which NSMase activity assay was performed according to experimental procedures. (**B**) MCF-7 cells were plated in 60 mm dishes and treated with different concentration of camptothecin. Cells were lysed 24 hours later and immunoblotted for nSMase2 and actin.

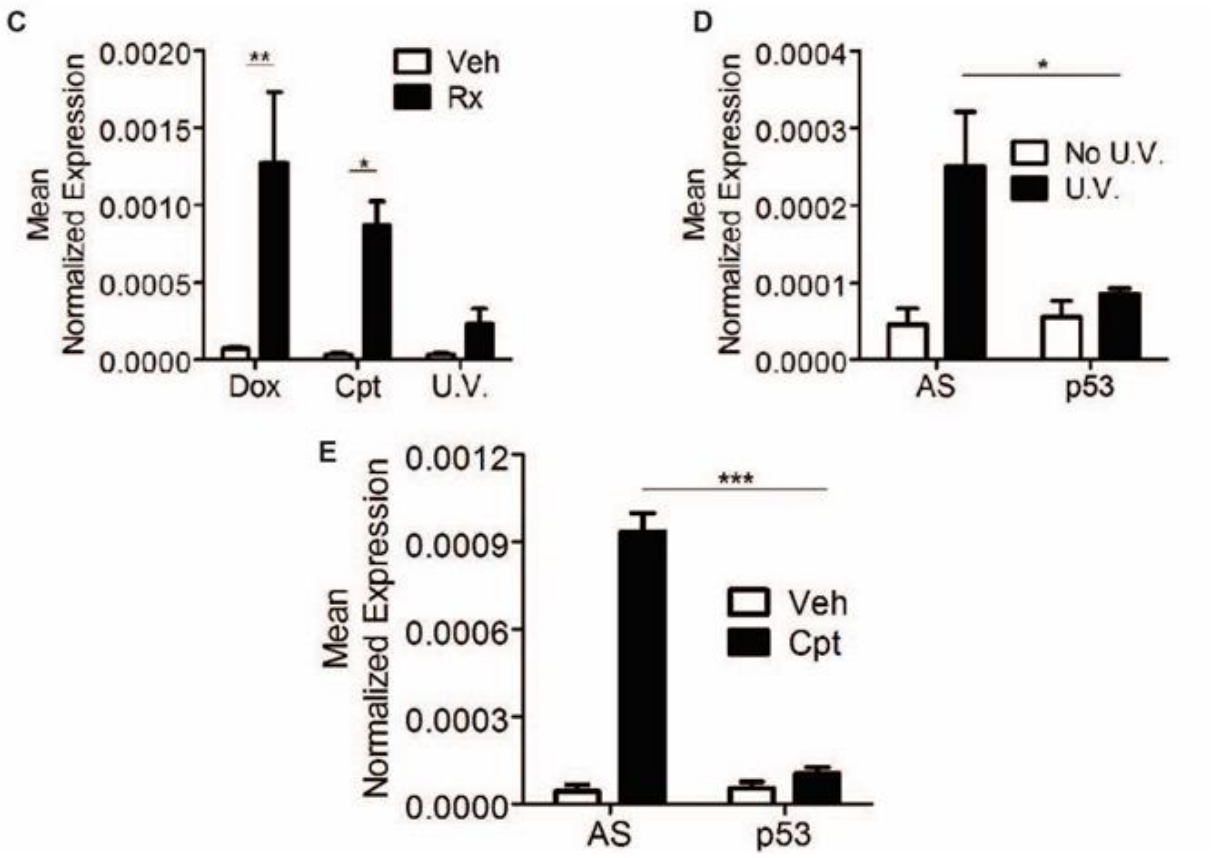


Figure 18 C-E: nSMase2 upregulation in response to DNA damage is specific to ssDNA breaks generating agents

(**C**) MCF-7 cells were plated in 60 mm dishes and treated with 0.6 μM doxorubicin, 0.8 μM camptothecin, 10 mJ/cm2 UV, Cells were lysed 24 hours later and RNA isolated and transformed to cdna and qRT-PCR for nSMAse2 was performed according to experimental procedures. (D-E) MCF-7 cells were plated in 60 mm dishes and siRNA to All Star negative control (AS) or p53 was performed for 24 hours. After that, cells were treated with vehicle, 10 mJ/cm2 UV (D), or 0.8 μM camptothecin (E). Cells were lysed 24 hours later and RNA isolated and transformed to cdna and qRT-PCR for nSMase2 was performed according to experimental procedures.

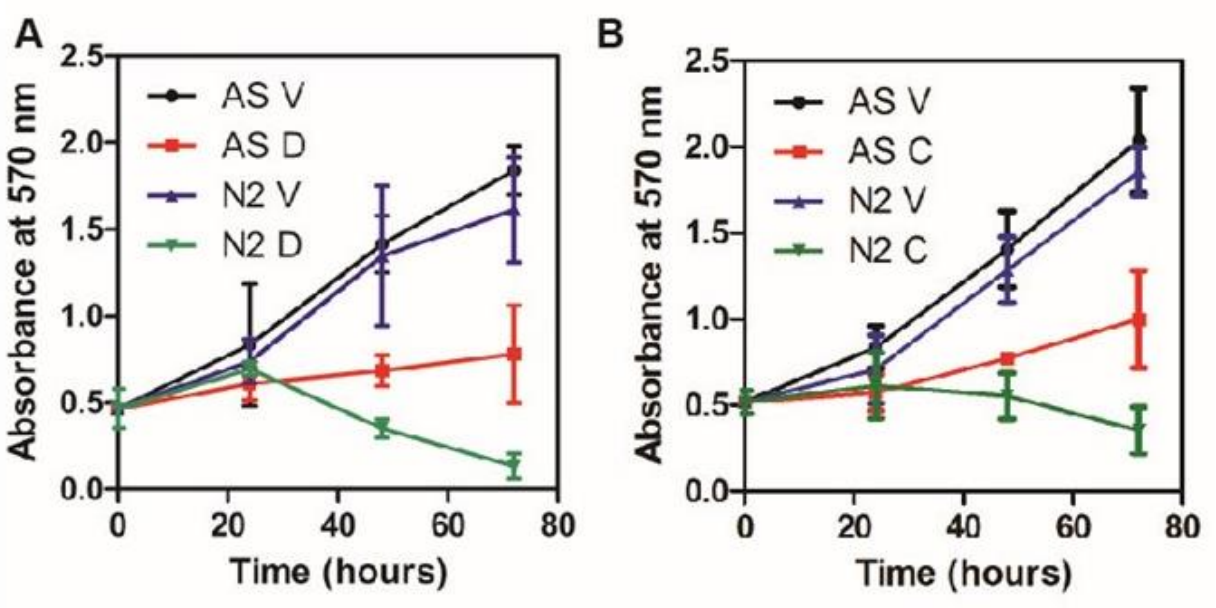


Figure 19 A-B: nSMase2 upregulation is protecting MCF-7 cells from apoptosis following ssDNA breaks

(**A-B**) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All start negative control (AS) or nSMase2 (N2). 24 hours later cells were treated with vehicle (AS V and N2 V) and 0.6 μM doxorubicin (AS D and N2 D) (**A**) or vehicle (AS V and N2 V) and 0.8 μM camptothecin (AS C and N2 C) (**B**) for 24, 48 and 72 hours and MTT assay was performed at each timepoint according to experimental procedures.

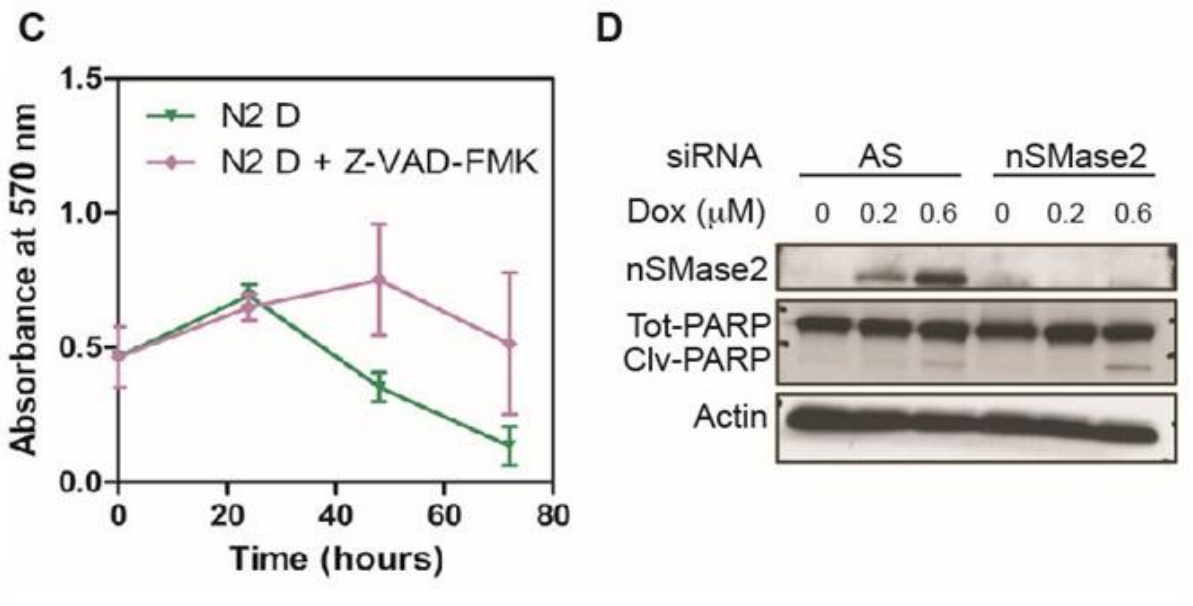


Figure 19 C-D: nSMase2 upregulation is protecting MCF-7 cells from apoptosis following ssDNA breaks

(C-D) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All star negative control (AS) or nSMase2 (N2). 24 hours later cells were treated with vehicle (AS V and N2 V) and 0.6 μM doxorubicin (AS D and N2 D). Prior to the doxorubicin treatment cells were pre-treated with vehicle or 10 μM Z-VAD-FMK for 1 hour. MTT assays were performed at the 24, 48 and 72 hours timepoints. (D) MCF-7 cells were seeded in 60 mm dishes and siRNA was performed to AS or nSMase2. Cells were then treated with different concentrations of doxorubicin for 24 hours, lysed and immunoblotted for nSMase2, PARP, and actin.

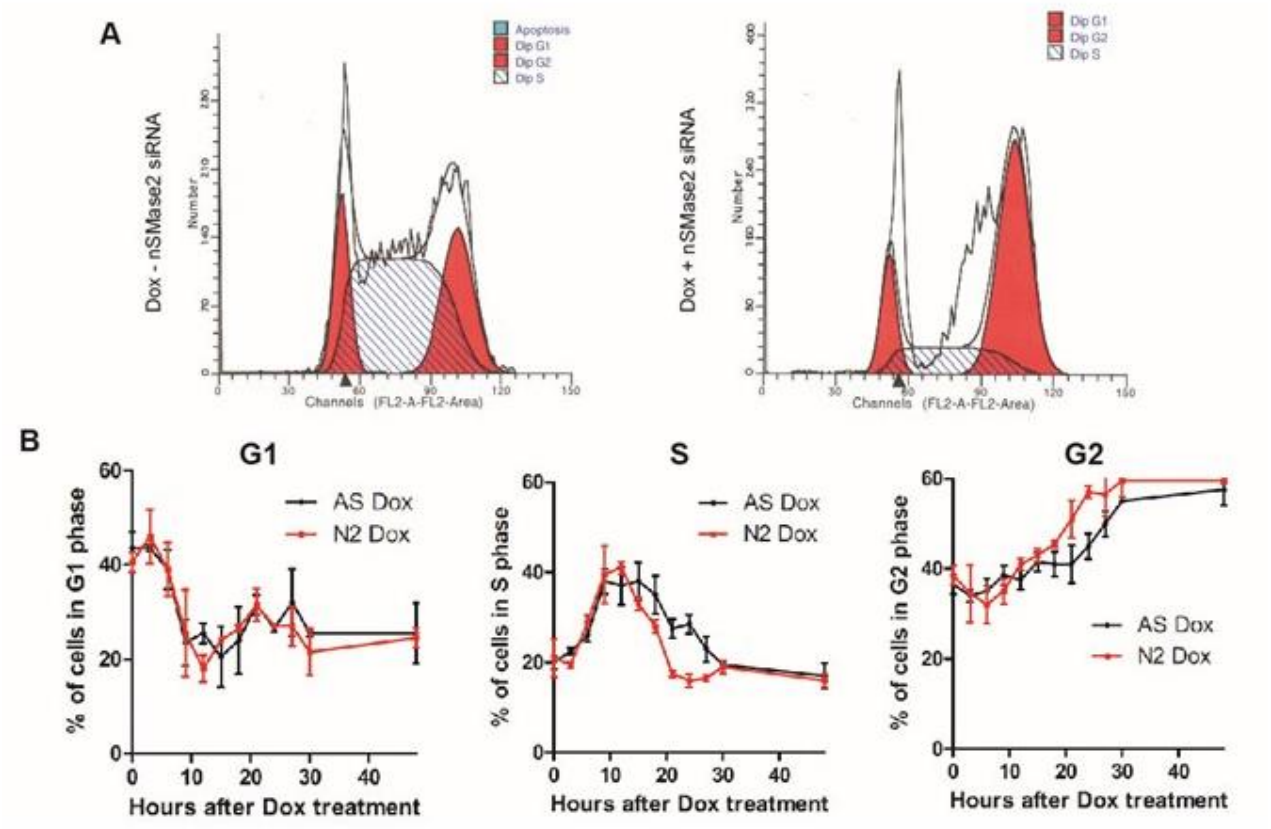


Figure 20 A-B: nSMase2 prolongs the duration of S phase following doxorubicin treatment.

(**A-B**) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All start negative control (AS) or nSMase2 (N2). 24 hours later cells were treated with vehicle (AS V and N2 V) and 0.6 μM doxorubicin (AS Dox and N2 Dox). Cells were collected either at 24 hours (**A**) or at different time points (**B**), fixed and analyzed for cell cycle distribution by PI staining according to the experimental procedures.

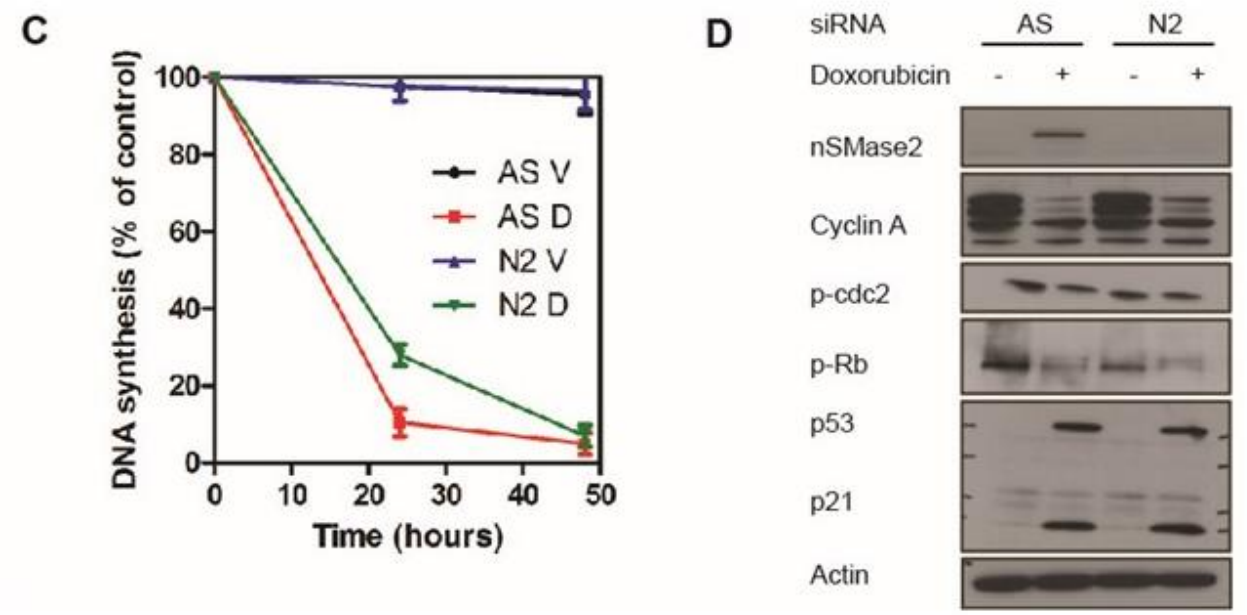


Figure 20 C-D: nSMase2 prolongs the duration of S phase following doxorubicin treatment.

(**A**) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All start negative control (AS) or nSMase2 (N2). 24 hours later cells were treated with vehicle (AS V and N2 V) and 0.6 μM doxorubicin (AS D and N2 D). Cells were pre-incubated in media containing C14 thymidine 1 hour prior to the treatment and pulsed with H3 thymidine for an hour before cell collection and radioresistant DNA synthesis assay performance according to experimental procedures. (**B**) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All start negative control (AS) or nSMase2 (N2). 24 hours later cells were treated with vehicle (AS V and N2 V) and 0.6 μM doxorubicin (AS D and N2 D). Cells were lysed and immunoblotted for nSMase2, cyclin A, p-cdc2 (Y14), p-Rb, p53, p21 and actin.

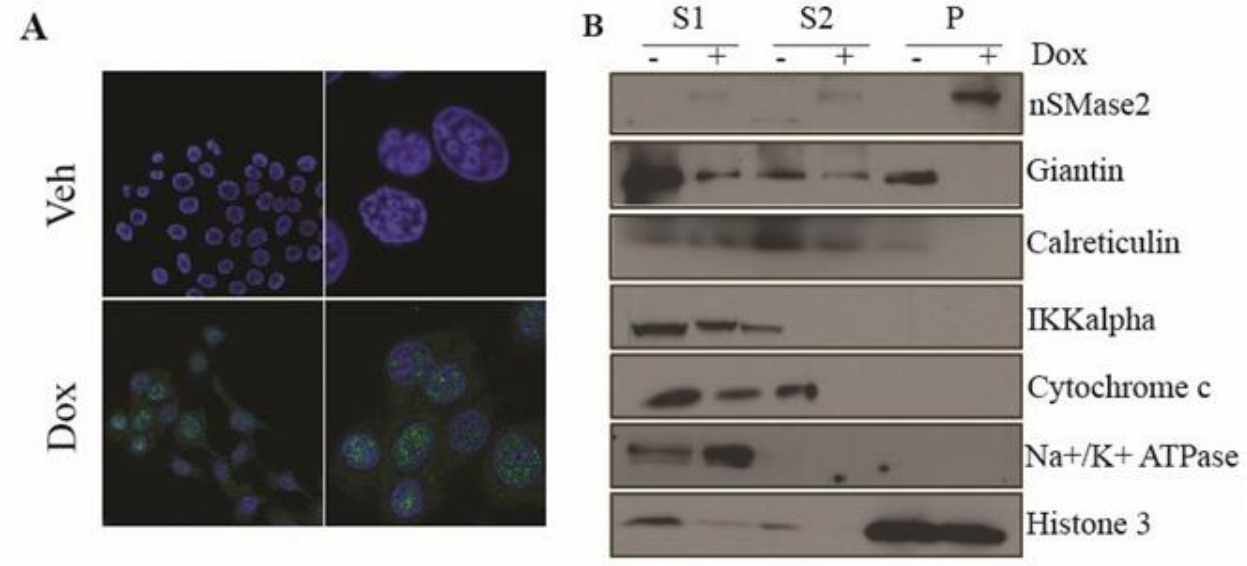


Figure 21 A-B: nSMase2 localizes to the nucleus following doxorubicin treatment

(**A**) MCF-7 cells were plated in 35 mm confocal dishes and treated with vehicle (veh) or 0.6 μM doxorubicin (Dox) for 24 hours. Cells were fixed with cold methanol, blocked and immunofluorescence was performed for DAPI (blue) and nSMase2 monoclonal antibody (green). (**B**) MCF-7 cells were seeded in 60 mm dishes and treated with vehicle or 0.6 μM doxorubicin for 24 hours after which cells were lysed and nuclear fractionation was performed. S1 and S2 indicates Sup1 and Sup2 while P indicates the pellet. Samples were immunoblotted for nSMase2, Giantin (marker of ER), Calreticulin (marker of Golgi), IKK alpha (cytoplasmic marker), cytochrome c (mitochondrial marker), NA+/K+ ATPase (plasma membrane), and histone 3 (nucleus).

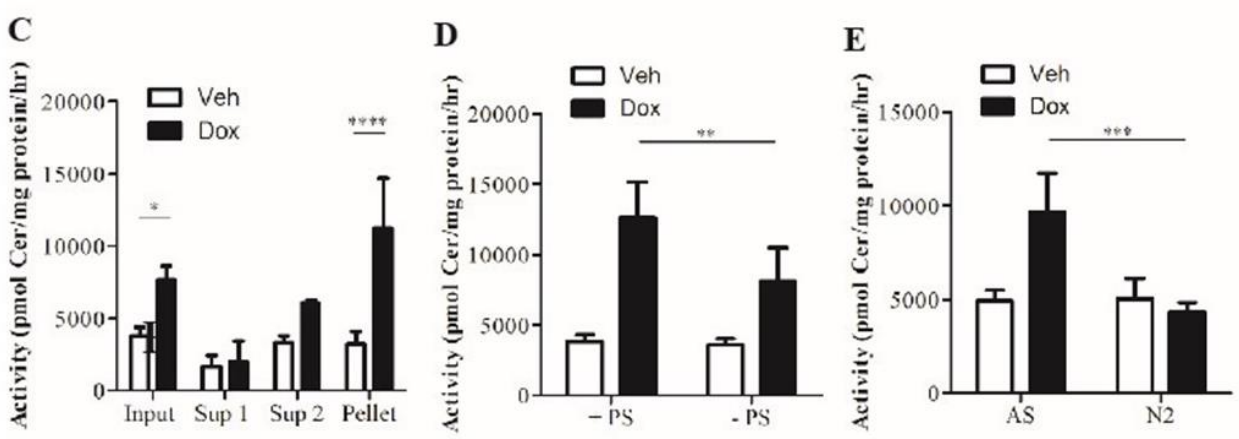


Figure 21 C-E: nSMase2 localizes to the nucleus following doxorubicin treatment

(**C**) MCF-7 cells were seeded in 60 mm dishes and treated with vehicle or 0.6 μM doxorubicin for 24 hours after which cells were lysed and nuclear fractionation was performed. On each fraction NSMase activity assay was performed, *P<0.05, **** p<0.0001. (**D**) MCF-7 cells were seeded in 60 mm dishes and treated with vehicle or 0.6 μM doxorubicin for 24 hours after which cells were lysed and nuclear fractionation was performed. NSMase activity assay was performed on the pellet on 2 assay buffers were prepared, one containing SM + PS and one containing SM only, **p<0.01. (**E**) MCF-7 cells were seeded in 60 mm dishes and siRNA to AS or to N2 was performed after which cells were treated with vehicle or 0.6 μM doxorubicin for 24 hours. Cells cells were then lysed and nuclear fractionation was performed. Activity assays were performed on the nuclear pellet, ***p<0.001.

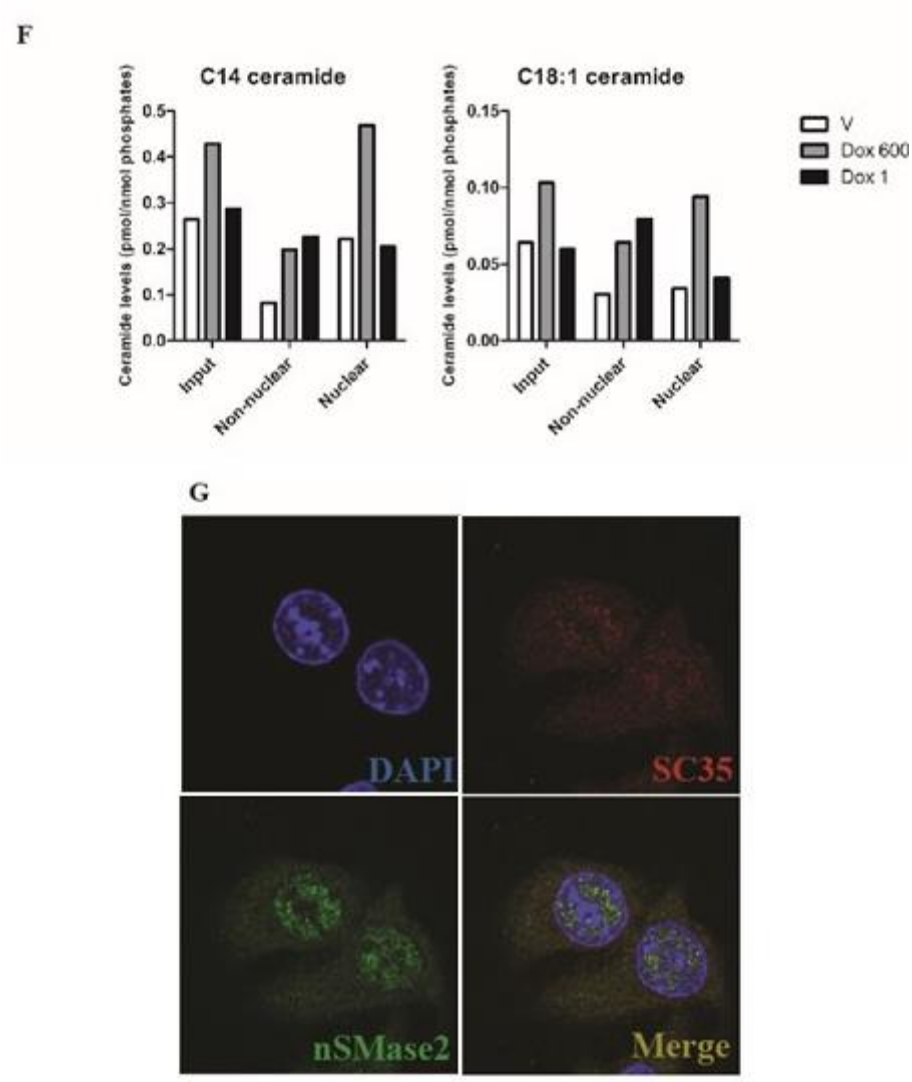


Figure 21 F-G: nSMase2 localizes to the nucleus following doxorubicin treatment

(**F**) MCF-7 cells were seeded in 100 mm dishes and treated with vehicle, 0.6 μM or 1μM doxorubicin for 24 hours after which cells were lysed and nuclear fractionation was performed. Fractions were sent to analysis by LC/MS. (**G**) MCF-7 cells were plated in 35 mm confocal dishes and treated with vehicle (veh) or 0.6 μM doxorubicin (Dox) for 24 hours. Cells were fixed with cold methanol, blocked and immunofluorescence was performed for DAPI (blue) and nSMase2 monoclonal antibody (green), or SC35, a marker of nuclear speckles (red).

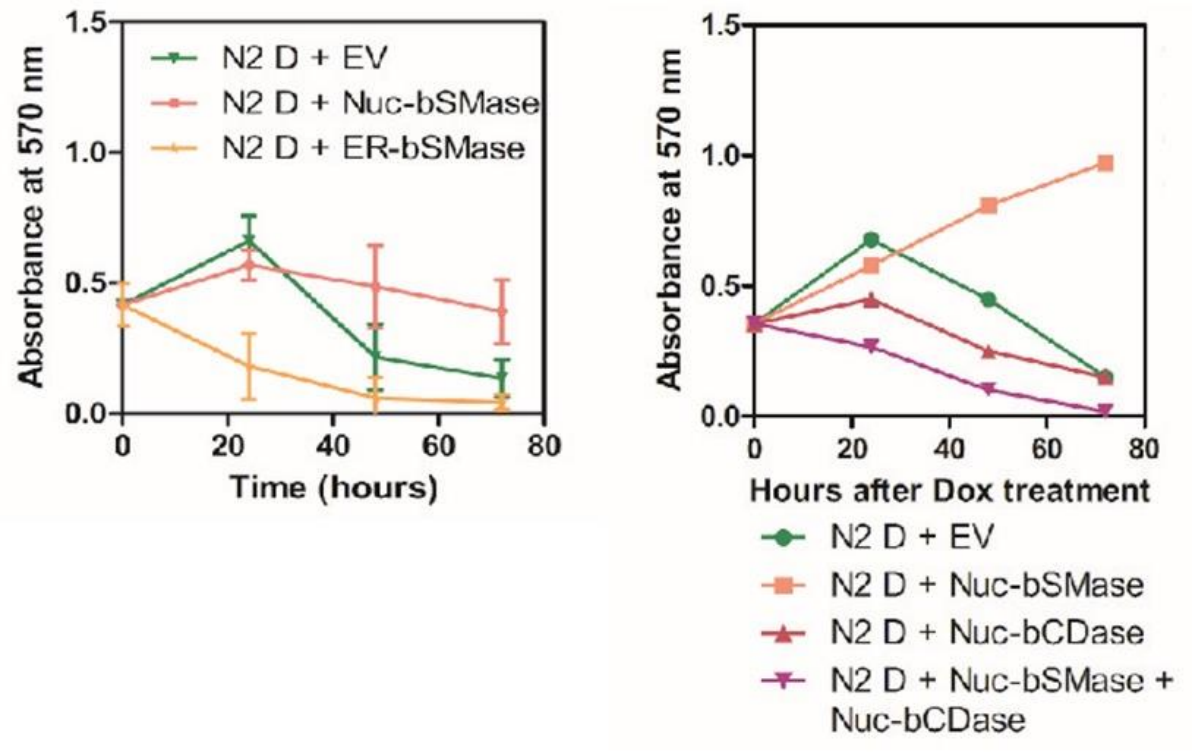


Figure 22 A-B: Nuclear C18:1 ceramide is the lipid responsible for nSMase2 effects

(**A-B**) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All start negative control (AS) or nSMase2 (N2). After that, empty vector (EV), nuclear-bSMase, ER-bSMase or Nuc-bCDase were overexpressed. 24 hours later cells were treated with vehicle (AS V and N2 V) and 0.6 □M doxorubicin (AS D and N2 D) (A) or vehicle (AS V and N2 V) 24, 48 and 72 hours and MTT assay was performed at each timepoint according to experimental procedures.

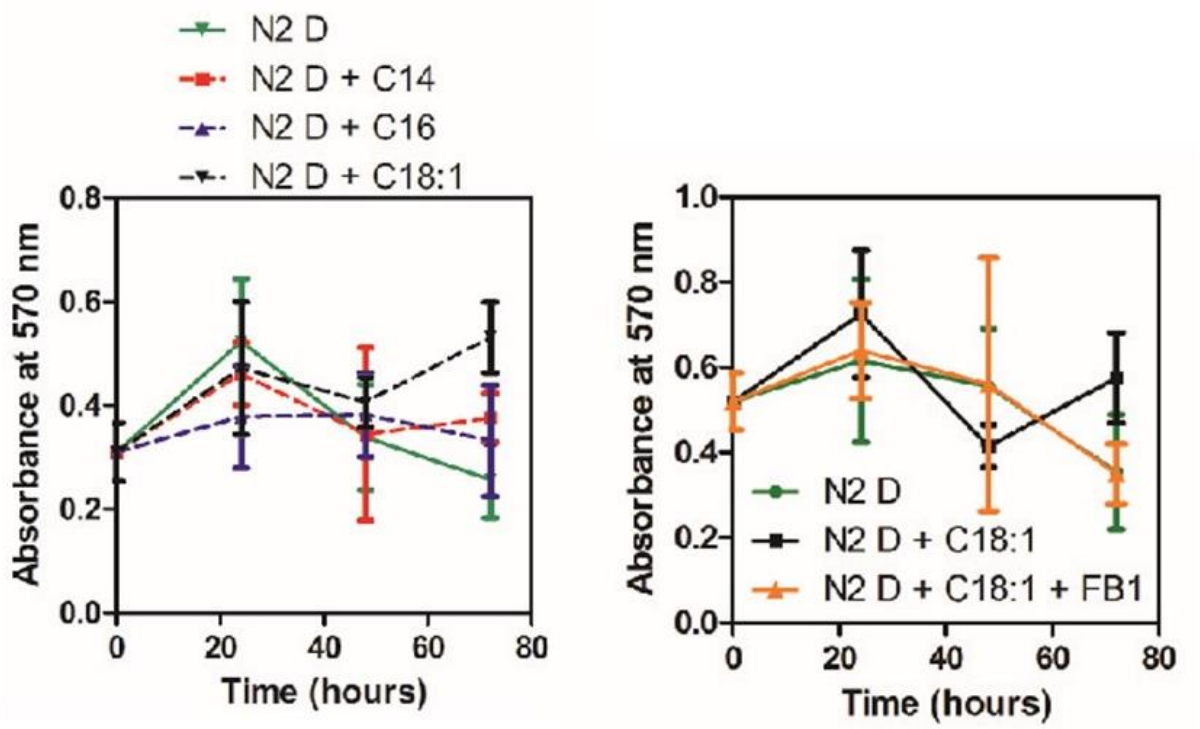


Figure 22 C-D: Nuclear C18:1 ceramide is the lipid responsible for nSMase2 effects

(**C**) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All start negative control (AS) or nSMase2 (N2). After that, cells were supplemeted with myristate, palmitate or oleate prior to treatment with vehicle or 0.6 μM doxorubicin. 24, 48 and 72 hours and MTT assay was performed at each timepoint according to experimental procedures. (**D**) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All start negative control (AS) or nSMase2 (N2). After that, cells were supplemeted with oleate or oleate and fumonisin (FB1) prior to treatment with vehicle or 0.6 μM doxorubicin. 24, 48 and 72 hours and MTT assay was performed at each timepoint according to experimental procedures.

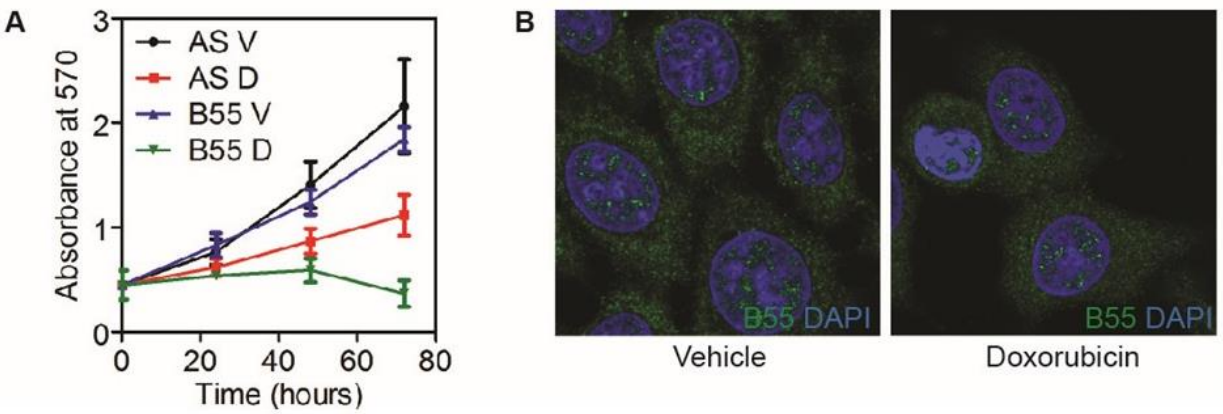


Figure 23 A-B: B55 is a potential downstream target of nSMase2

(**A**) MCF-7 cells were seeded in 6 well plates and siRNA was performed to either All start negative control (AS) or B55. 24 hours later cells were treated with vehicle (AS V and B55 V) and 0.6 μM doxorubicin (AS D and B55 D) for 24, 48 and 72 hours and MTT assay was performed at each timepoint according to experimental procedures. (**B**) MCF-7 cells were seeded in 35 mm confocal dishes and treated with vehicle or doxorubicin. Cells were fixed and immunofluorescence was done for B55 and DAPI.

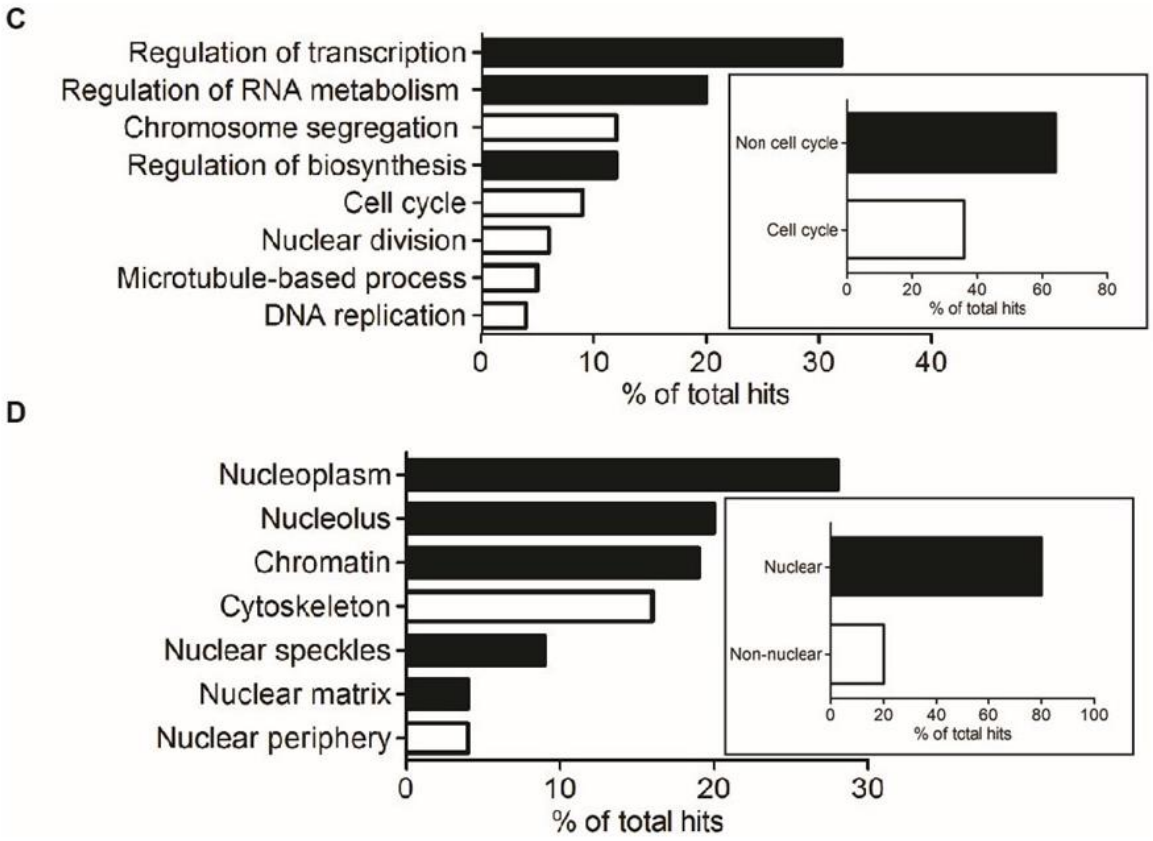


Figure 23 C-D: B55 is a potential downstream target of nSMase2

(**C-D**) Shown are the dephosphorylation hits classified on david software by biological processes (**C**) and cellular localization (**D**)

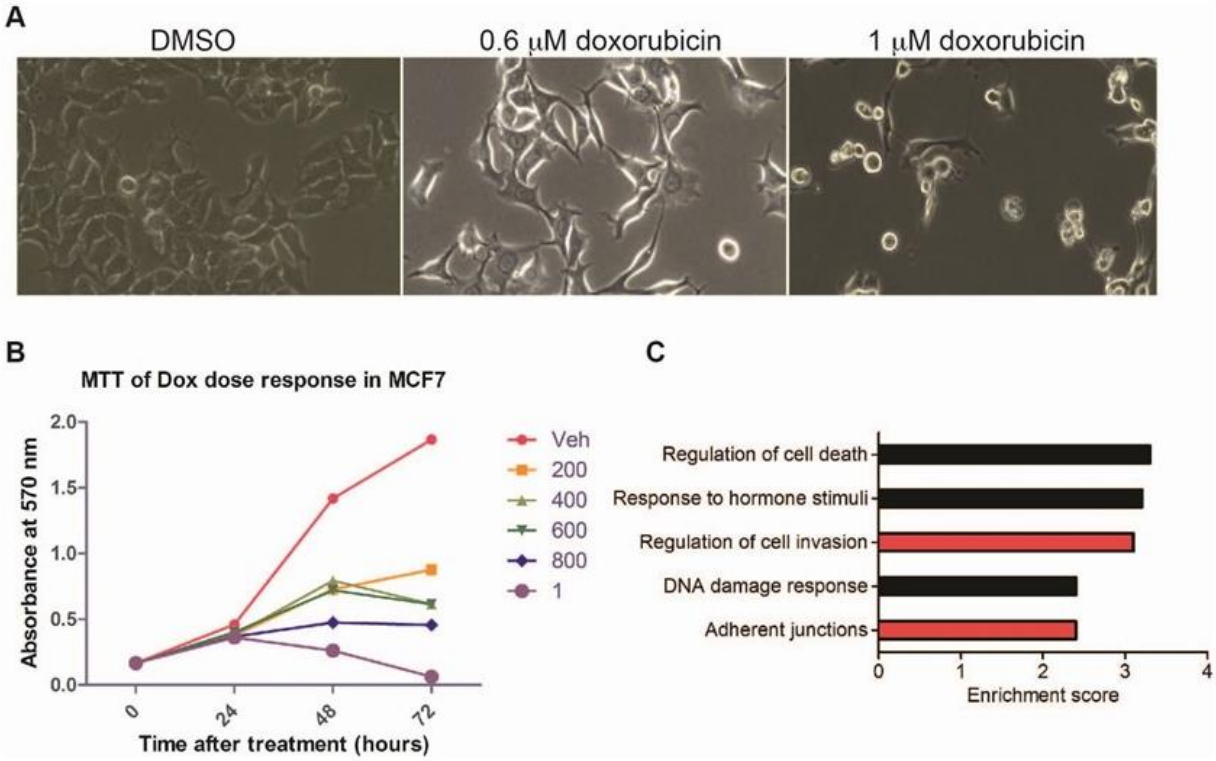


Figure 24 A-C: Doxorubicin induces invasive pathways at sublethal doses in MCF-7

(A) MCF-7 cells were plated in 60 mm dishes and treated with either vehicle (DMSO) or different concentrations of doxorubicin for 24 hours after which pictures were taken using a light microscope. (**B**). MCF-7 cells were plated in 6 well plates and treated with different concentrations of doxorubicin for 24 hours after which MTT assays for cell viability were performed according to "Experimental procedures". (**C**) MCF-7 cells were plated in 60 mm dishes and treated with vehicle or 0.6 μM doxorubicin for 24 hours. RNA was then isolated and sent for RNA sequencing as described under "Experimental procedures". Data were plotted on DAVID software and enrichment scores were calculated for the genes upregulated by 2 fold or more. Shown in red are invasive pathways.

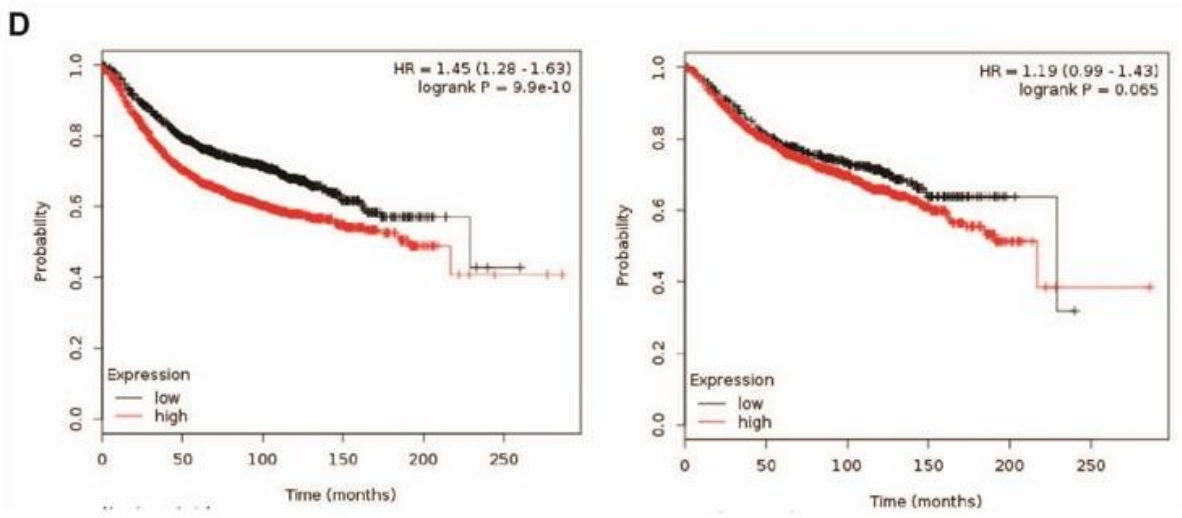


Figure 24 D: Doxorubicin induces invasive pathways at sublethal doses in MCF-7

(D) Kaplan-Meier plots of survival of breast cancer patients expressing low and high Src with chemotherapy (1st panel) and without chemotherapy (2nd panel)

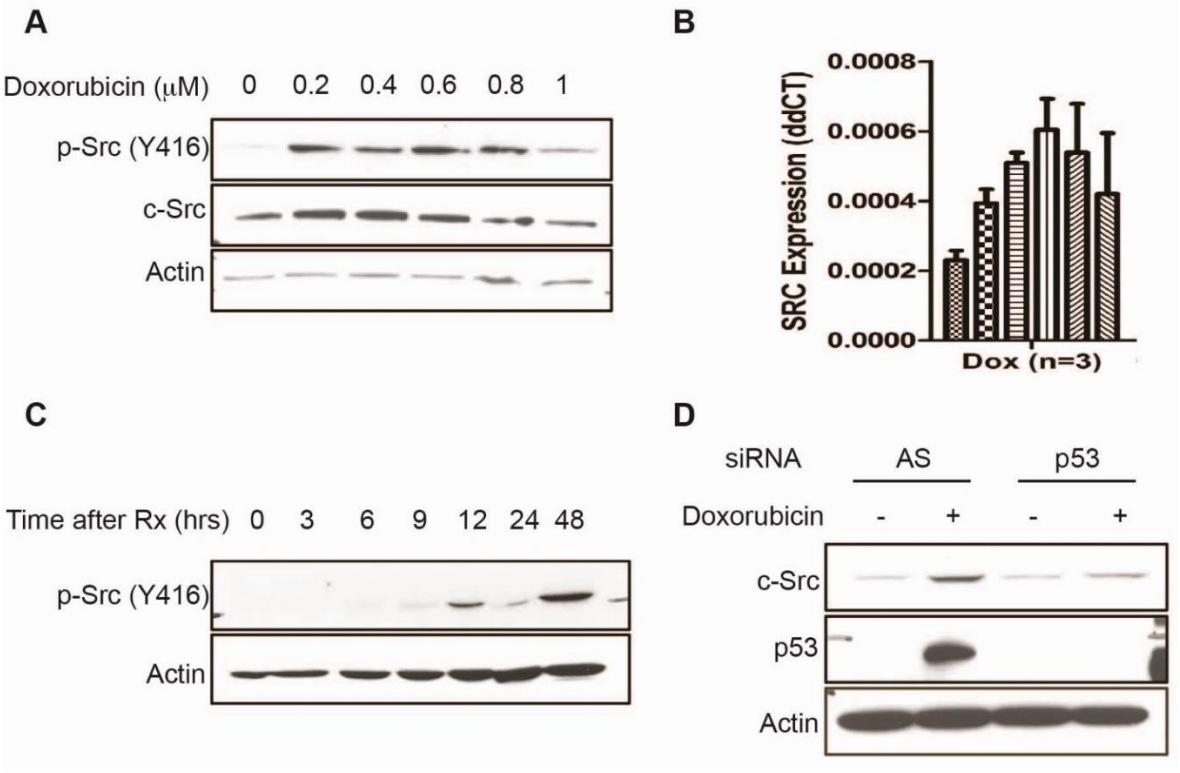


Figure 25: Sublethal doxorubicin induces Src in a p53-dependent manner

(**A-B**) MCF-7 cells well plated in 60 mm dishes and treated with different doses of doxorubicin. After 24 hours, cells were collected for immunoblotting for p-Src (Y416), c-Src and actin (**A**) or RNA was isolated and transformed to cDNA after which qRT PCT was run for c-Src (**B**). (**C)** MCF-7 cells were seeded in 60 mm dishes and were treated with 0.6 μM doxorubicin for different time points. Cells were collected and immunoblotted for p-Src (Y416) and actin. (**D**) MCf-7 cells were plated in 60 mm dishes and transfected with either All Star negative control (AS) siRNa or p53 siRNA. 24 hours later cells were treated with doxorubicin for 24 hours after which cells were collected and immunoblotted for c-Src, p53 and acting.

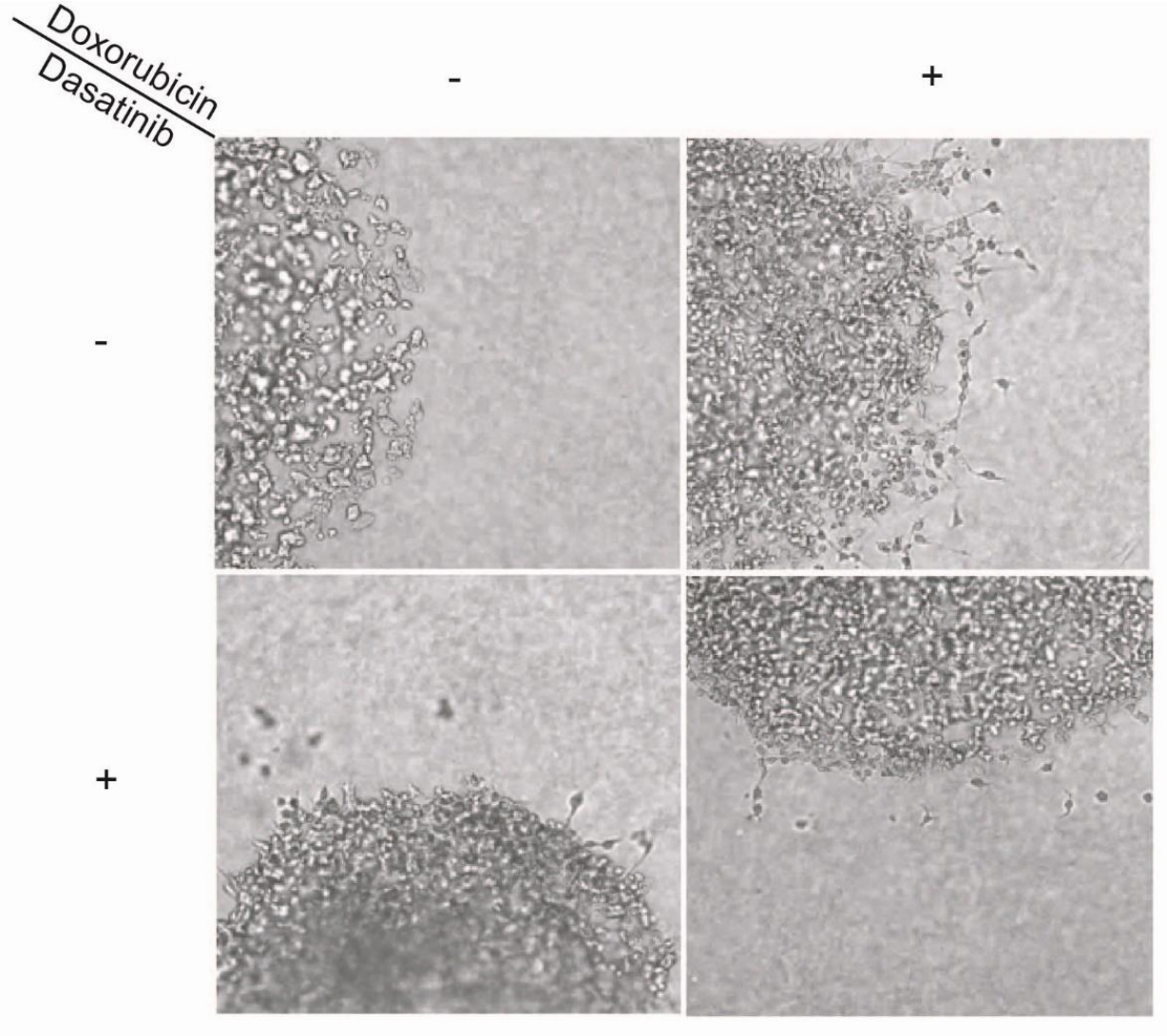


Figure 26: Dasatinib inhibits doxorubicin-induced migration

MCF-7 cells were seeded in 60 mm dishes and treated with vehicle or doxorubicin. Prior to each treatment, 100 nM of dasatinib was added. Collagen matrix assay was performed and pictures were taken with an EVOS microscope.

Supplementary figures

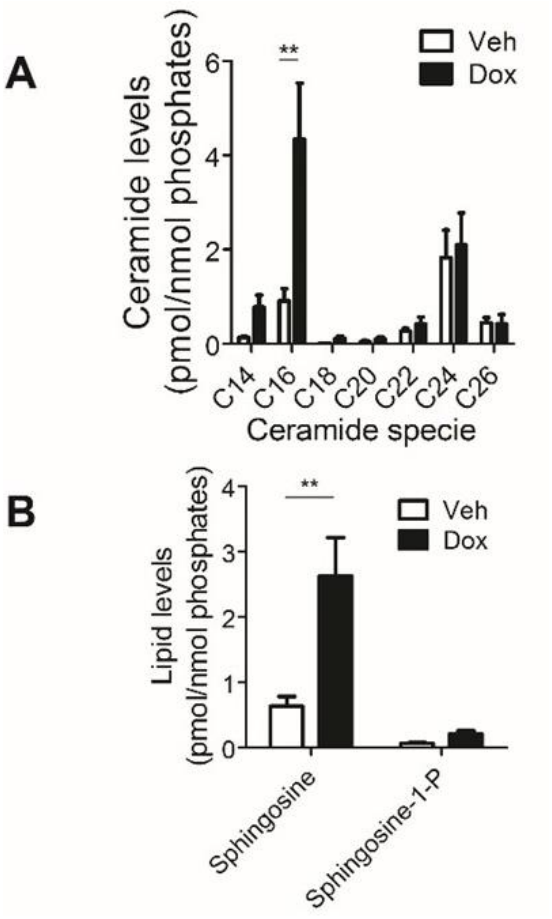


Figure S1: Sphingolipid levels following doxorubicin treatment

MCF-7 cells were collected 24 hours after treatment and analyzed for sphingolipids by LC/MS mass spectrometry. Shown are ceramide species levels (A), sphingosine levels and sphingosine-1-phosphate levels (B), **p<0.01

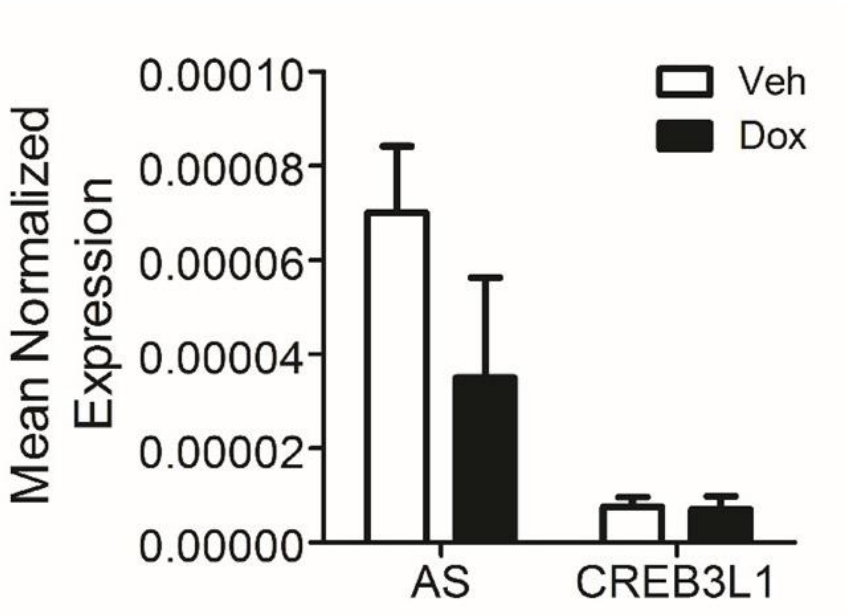
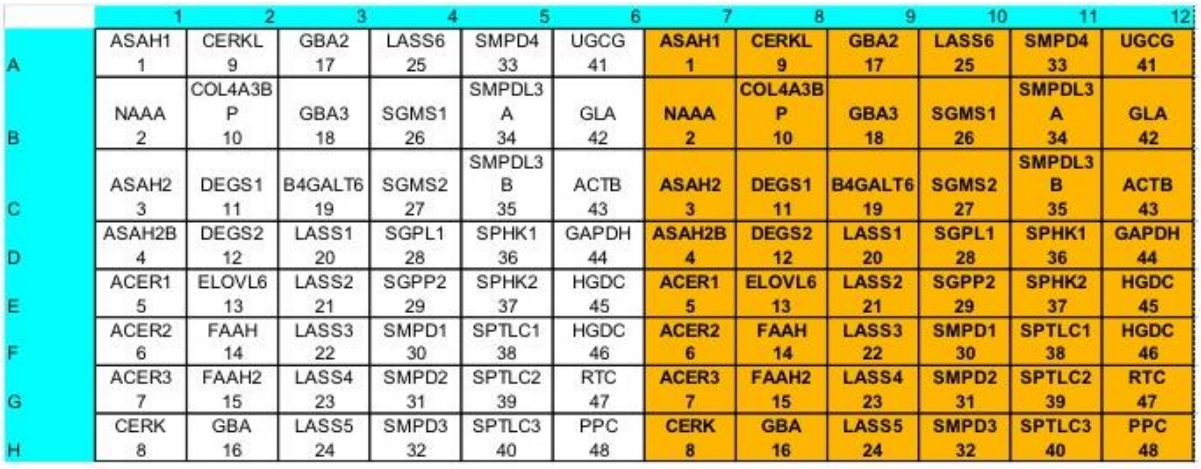


Figure S2: Verification of CREB3L1 knockdown

MCF-7 cells were seeded in 60 mm dishes and siRNA knockdown was performed using AS or CREB3L1 for 24 hours. After that, vehicle or doxorubicin were added, cells were collected, RNA was isolated and transformed to cDNA and qRT-PCR was performed for CREB3L1.



	1	2	3	4	5	6	7	8	9	10	11	12
A	ASAH1 1	CERKL 9	GBA2 17	LASS6 25	SMPD4 33	UGCG 41	ASAH1 1	CERKL 9	GBA2 17	LASS6 25	SMPD4 33	UGCG 41
B	NAAA 2	COL4A3BP 10	GBA3 18	SGMS1 26	SMPDL3A 34	GLA 42	NAAA 2	COL4A3BP 10	GBA3 18	SGMS1 26	SMPDL3A 34	GLA 42
C	ASAH2 3	DEGS1 11	B4GALT6 19	SGMS2 27	SMPDL3B 35	ACTB 43	ASAH2 3	DEGS1 11	B4GALT6 19	SGMS2 27	SMPDL3B 35	ACTB 43
D	ASAH2B 4	DEGS2 12	LASS1 20	SGPL1 28	SPHK1 36	GAPDH 44	ASAH2B 4	DEGS2 12	LASS1 20	SGPL1 28	SPHK1 36	GAPDH 44
E	ACER1 5	ELOVL6 13	LASS2 21	SGPP2 29	SPHK2 37	HGDC 45	ACER1 5	ELOVL6 13	LASS2 21	SGPP2 29	SPHK2 37	HGDC 45
F	ACER2 6	FAAH 14	LASS3 22	SMPD1 30	SPTLC1 38	HGDC 46	ACER2 6	FAAH 14	LASS3 22	SMPD1 30	SPTLC1 38	HGDC 46
G	ACER3 7	FAAH2 15	LASS4 23	SMPD2 31	SPTLC2 39	RTC 47	ACER3 7	FAAH2 15	LASS4 23	SMPD2 31	SPTLC2 39	RTC 47
H	CERK 8	GBA 16	LASS5 24	SMPD3 32	SPTLC3 40	PPC 48	CERK 8	GBA 16	LASS5 24	SMPD3 32	SPTLC3 40	PPC 48

Figure S3: Custom qRT-PCR array for sphingolipid genes

This qRT PCR array contains a set of 48 genes in duplicate. Normalization is done to both actin and GAPDH.

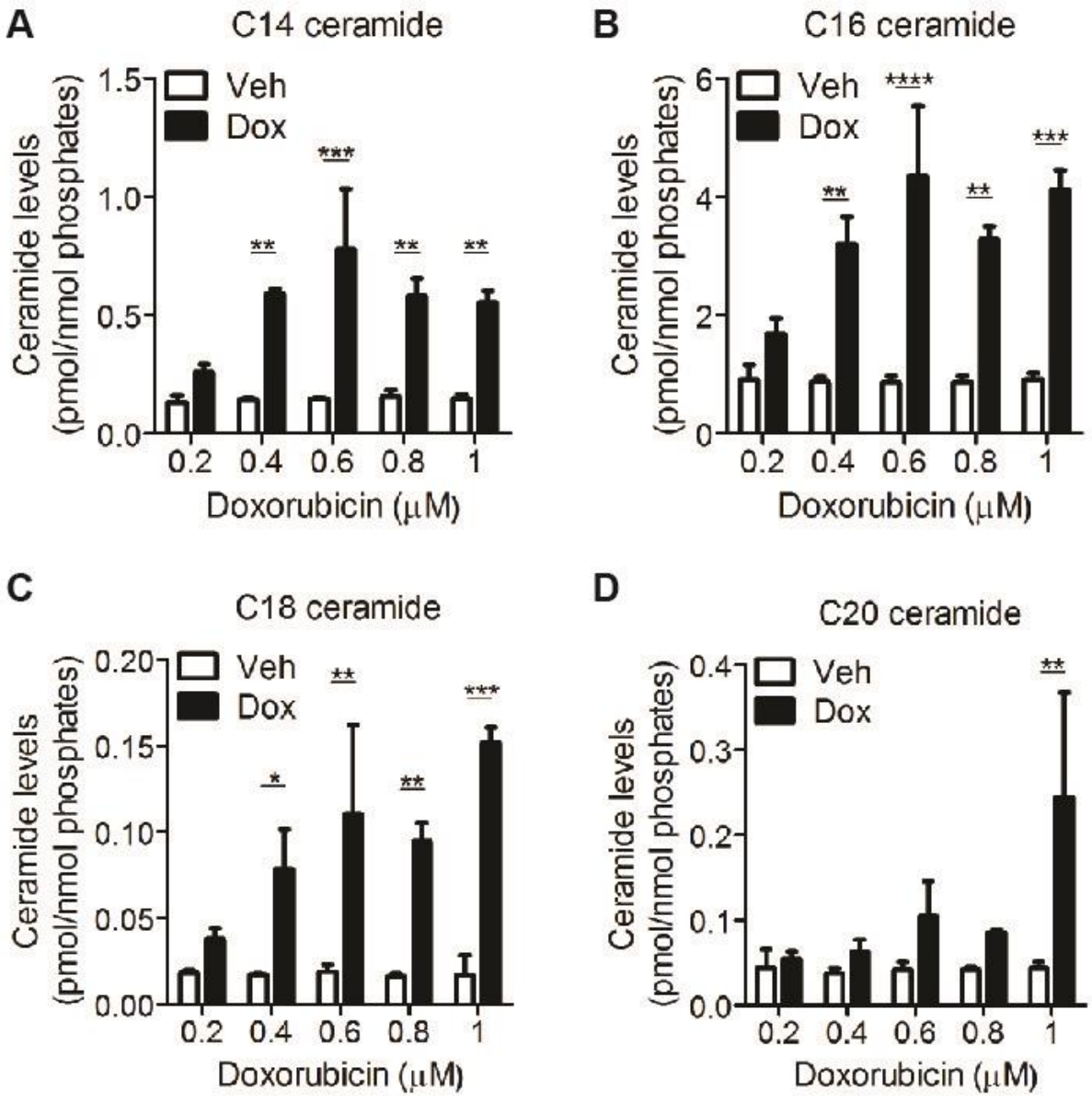


Figure S4 A-D: Dose dependent changes in ceramide species

(**A-D**) MCF-7 cells were seeded in 100 mm dishes and treated with different doses of doxorubicin. 24 hours later cells were harvested in cell extraction medium and sent for the lipid mass spectroscopy core for LC/MS according to experimental procedures, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

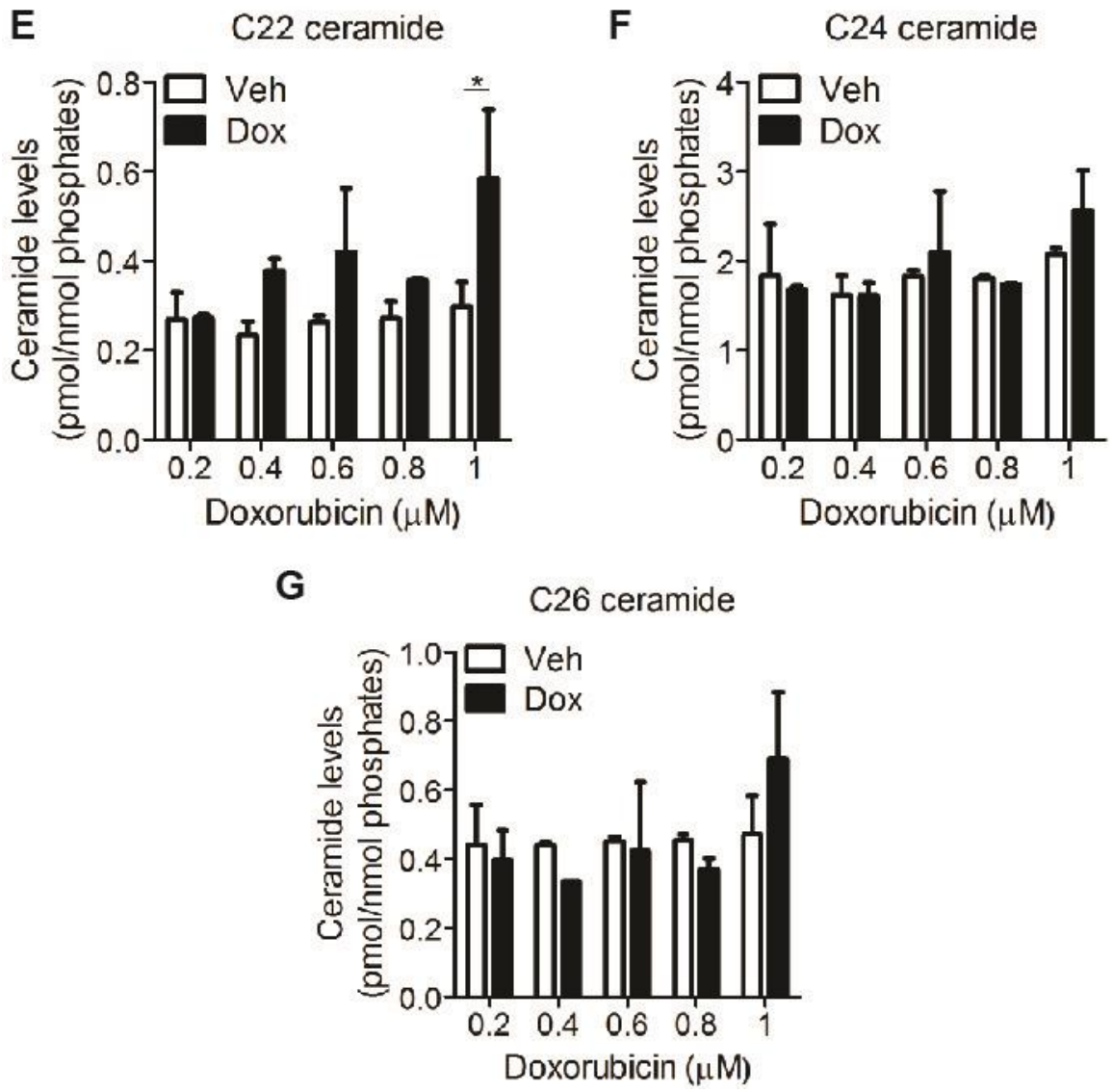


Figure S4 E-G: Dose dependent changes in ceramide species

(**A-D**) MCF-7 cells were seeded in 100 mm dishes and treated with different doses of doxorubicin. 24 hours later cells were harvested in cell extraction medium and sent for the lipid mass spectroscopy core for LC/MS according to experimental procedures, *$p<0.05$

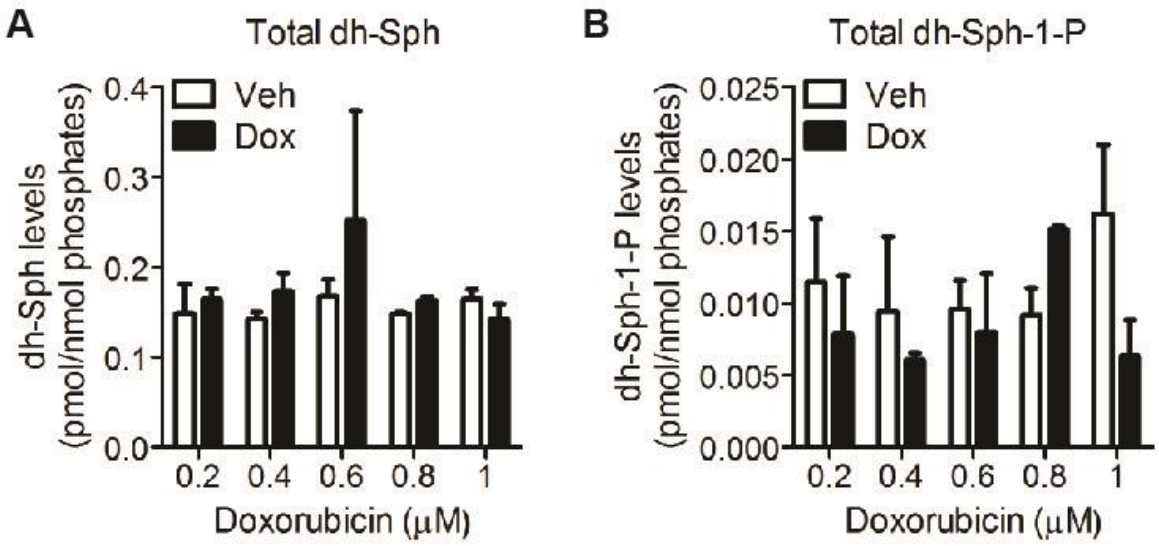


Figure S5: Dose dependent changes in dihydrosphingosine and dihysphingosine-1-phosphate

(**A-D**) MCF-7 cells were seeded in 100 mm dishes and treated with different doses of doxorubicin. 24 hours later cells were harvested in cell extraction medium and sent for the lipid mass spectroscopy core for LC/MS according to experimental procedures.

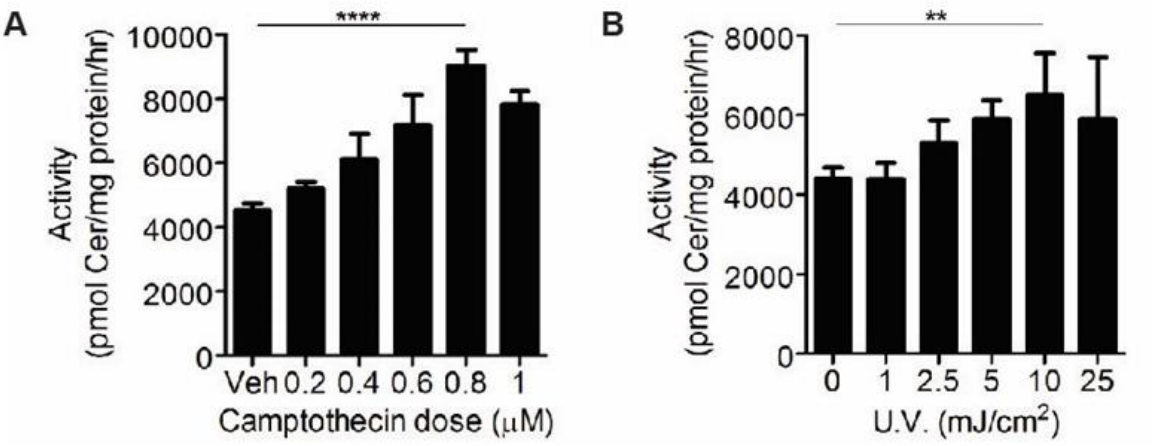


Figure S6 A-B: NSMase activity following dose responses of DNA damaging agents

(**A-B**). MCF-7 cells were seeded in 60 mm plates and treated with different concentrations of camptothecin (**A**) or different doses of UV (**B**) for 24 hours after which cells were lysed and NSMase activity assay was performed.

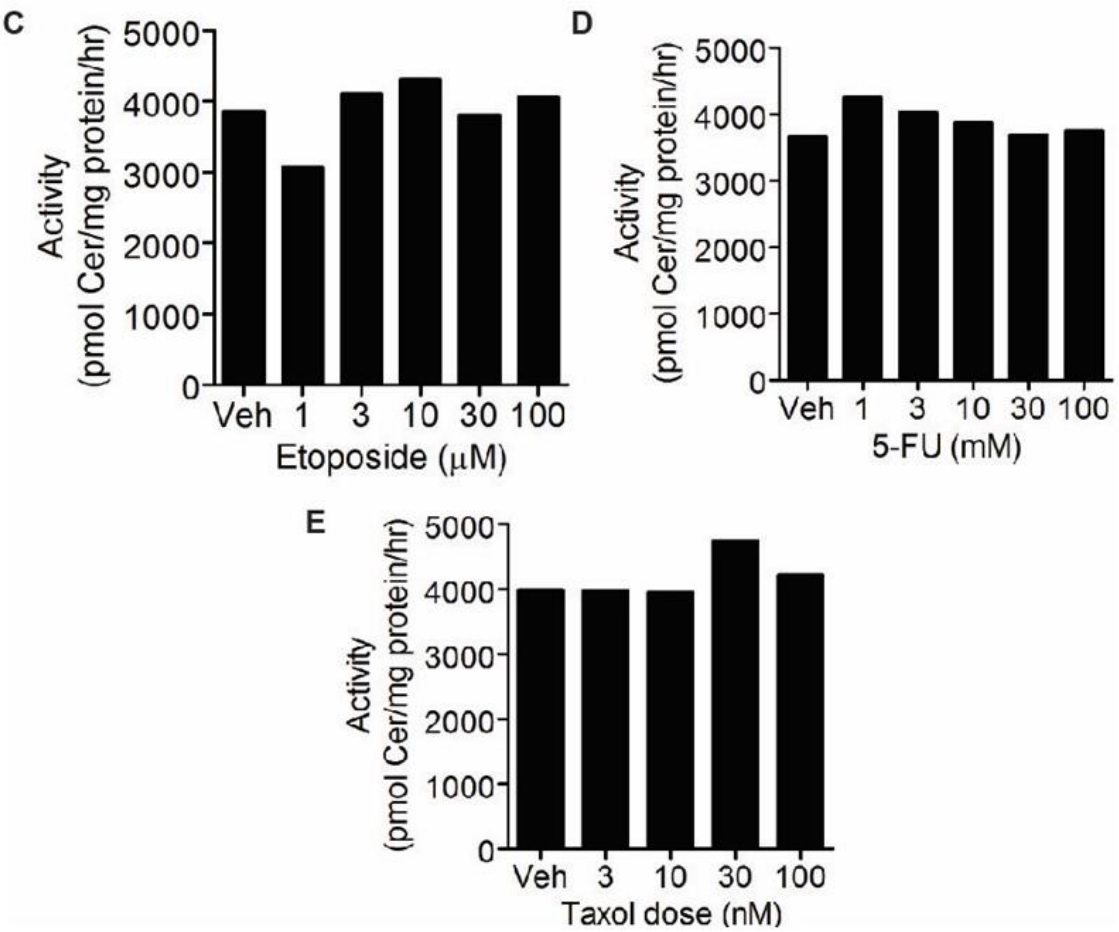


Figure S6 C-E: NSMase activity following dose responses of DNA damaging agents

(**A-B**). MCF-7 cells were seeded in 60 mm plates and treated with different concentrations of etoposide (**C**), 5-FU (**D**), or taxol (**E**) for 24 hours after which cells were lysed and NSMase activity assay was performed.

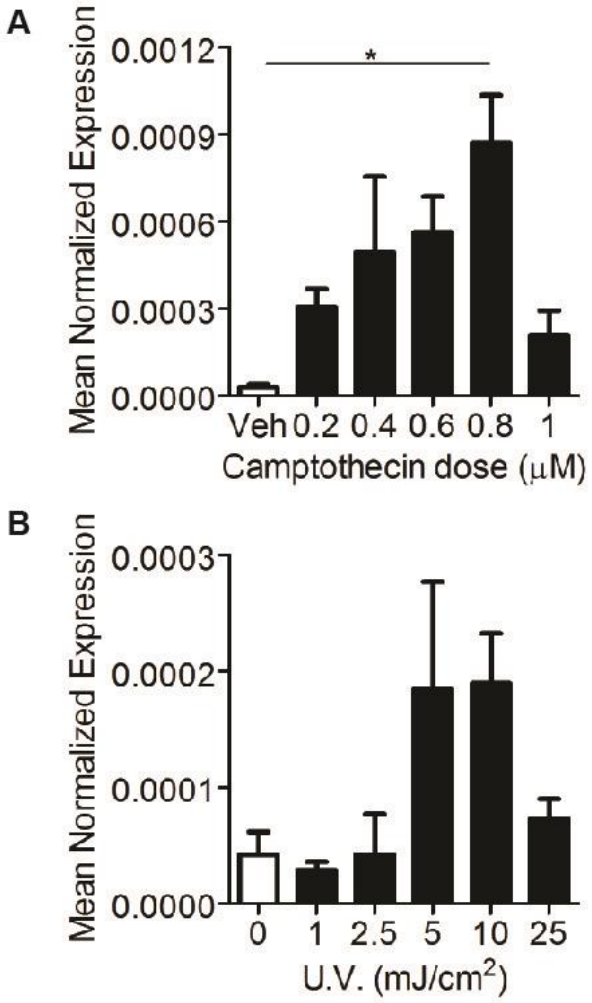


Figure S7: NSMase2 message levels following dose responses of DNA damaging agents

(**A-B**). MCF-7 cells were seeded in 60 mm plates and treated with different concentrations of camptothecin (**A**) or different doses of UV (**B**) for 24 hours after which cells were lysed, RNA was isolated and transformed to cDNA and qRT-PCR was run for nSMase2.

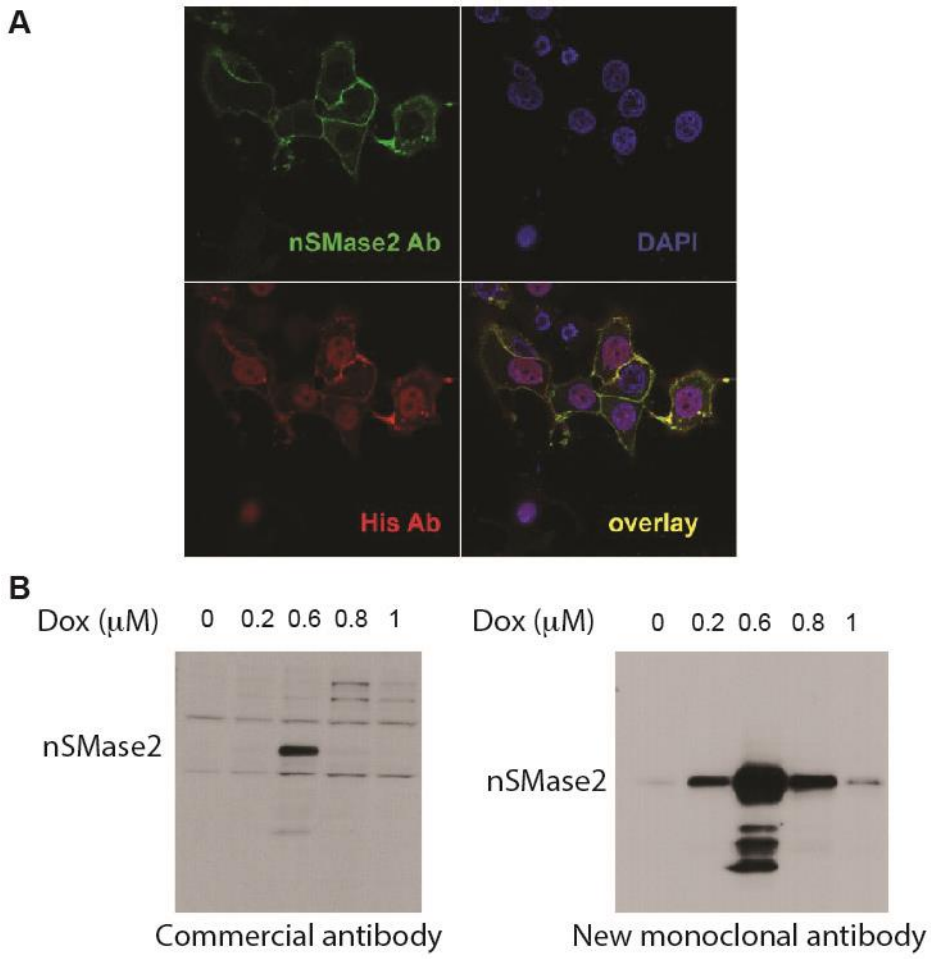


Figure S8: Validation of new nSMase2 monoclonal antibody

(**A**) MCF-7 stables overexpressing nSMase2 V5/His were plated in 35 mm confocal dishes for 24 hours after which they were fixed and immunofluorescence was done for nSMase2, His and DAPI. (**B**) MCF-7 cells were seeded in a 60 mm dishes after which they were treated with different doses of doxorubicin for 24 hours and immunoblotted for nSMase2 using nSMase2 polyclonal antibody from Santa Cruz and nSMase2 monoclonal antibody developed in the lab.

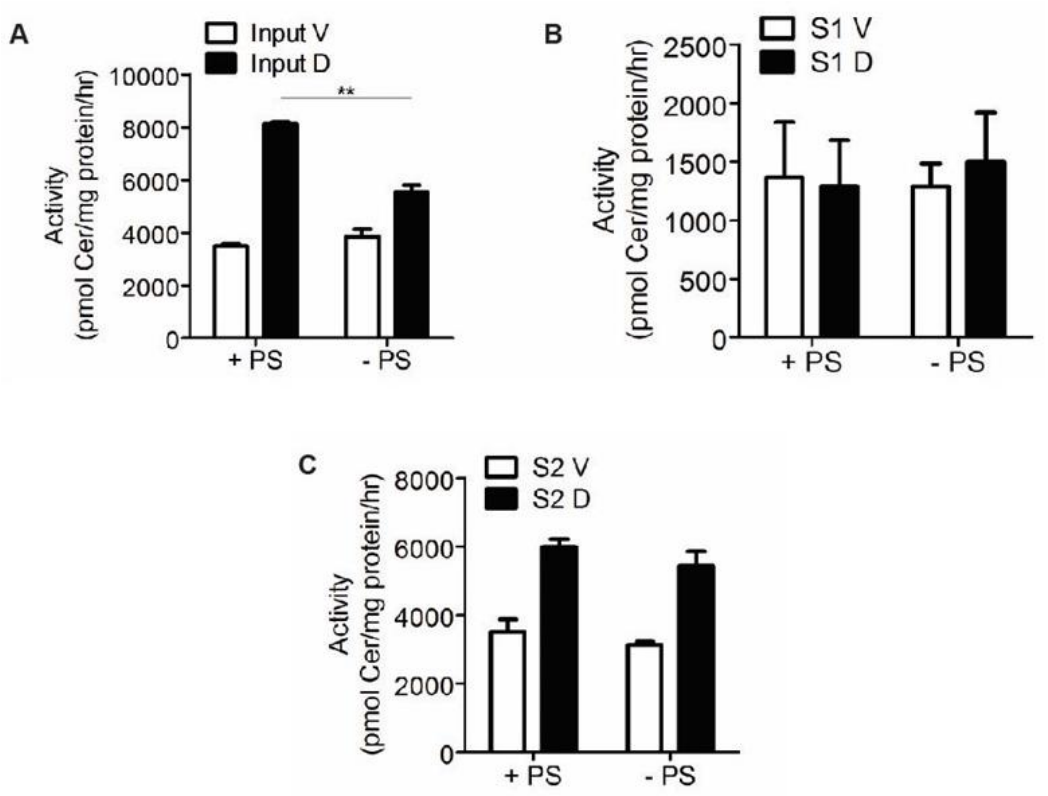


Figure S9 A-C: NSMase activity assays on cellular fractions with doxorubicin

(A-C). MCF-7 cells were seeded in 60 mm dishes and treated with vehicle or 0.6 μM doxorubicin for 24 hours after which cells were lysed and nuclear fractionation was performed. On each fraction NSMase activity assay was performed with and without PS in the assay buffer. Input (**A**), Sup 1 (S1) (**B**) and Sup2 (S2) (**C**) **P<0.01.

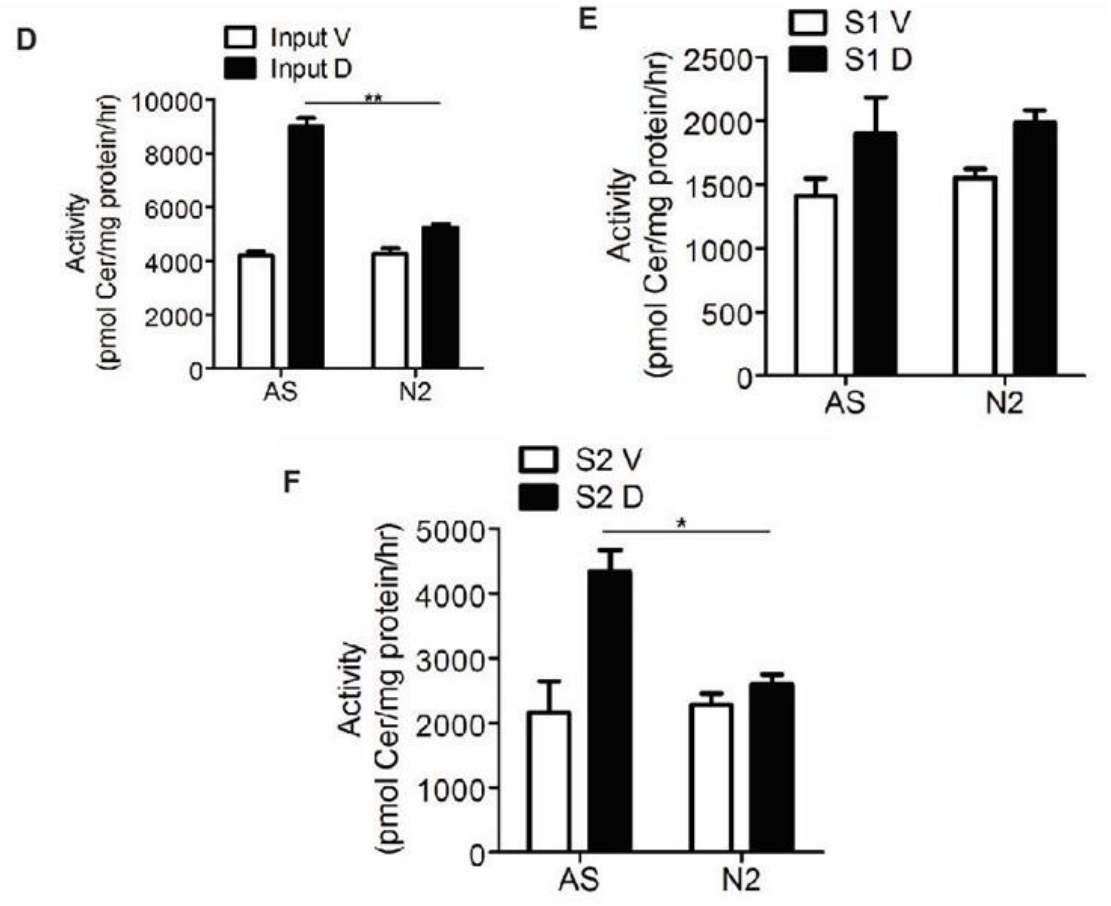


Figure S9 D-F: NSMase activity assays on cellular fractions with doxorubicin

(A-C). MCF-7 cells were seeded in 60 mm dishes and siRNA transfections were performed to AS and N2 after which cells were treated with vehicle or 0.6 μM doxorubicin for 24 hours. Cells were lysed and nuclear fractionation was performed. On each fraction NSMase activity assay was performed. Input (**D**), Sup 1 (S1) (**E**) and Sup2 (S2) (**F**), *P<0.05 **P<0.01.

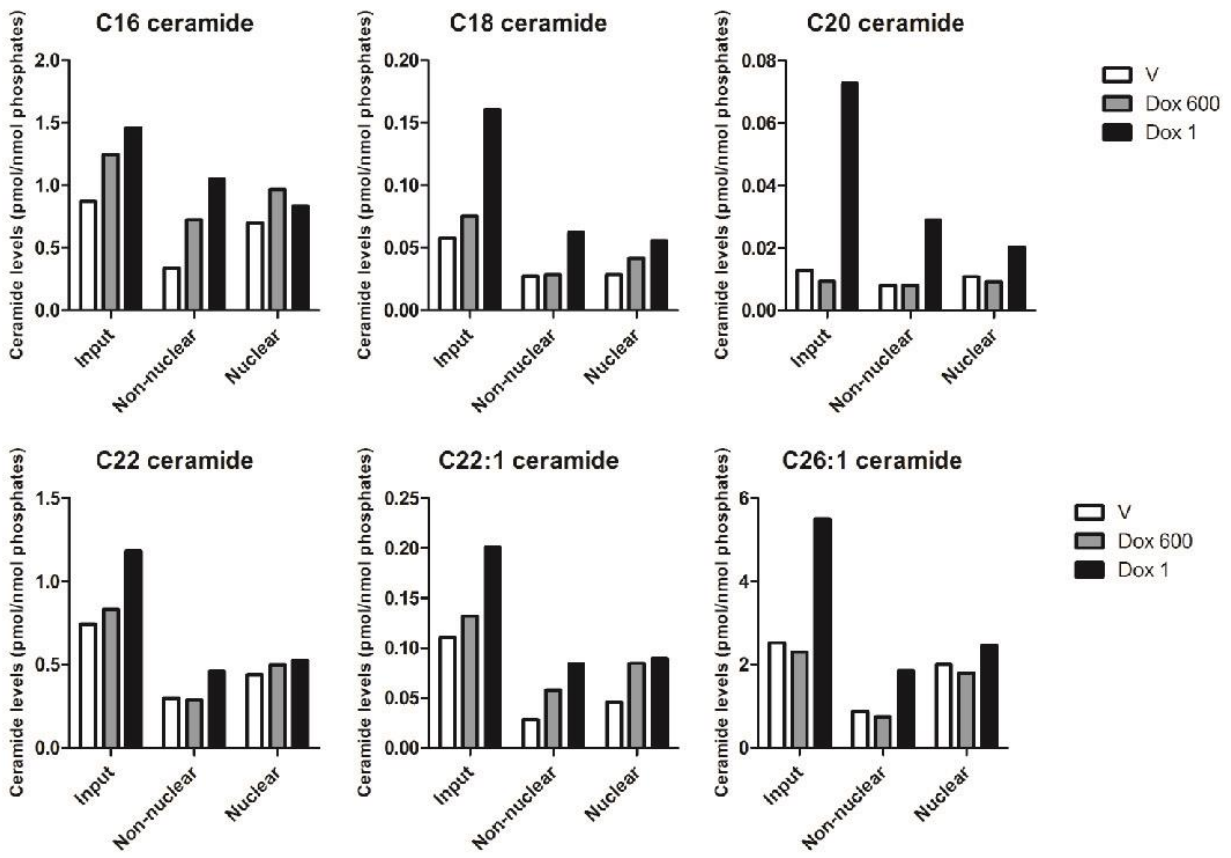


Figure S10: Increased ceramide species at 1μM doxorubicin

MCF-7 cells were plated in 100 mm dishes and were treated with either vehicle or different concentrations of doxorubicin for 24 hours. Following that lipids were extracted and send for analysis by LC/MS. Shown are the changes in ceramide species mostly at 1μM.

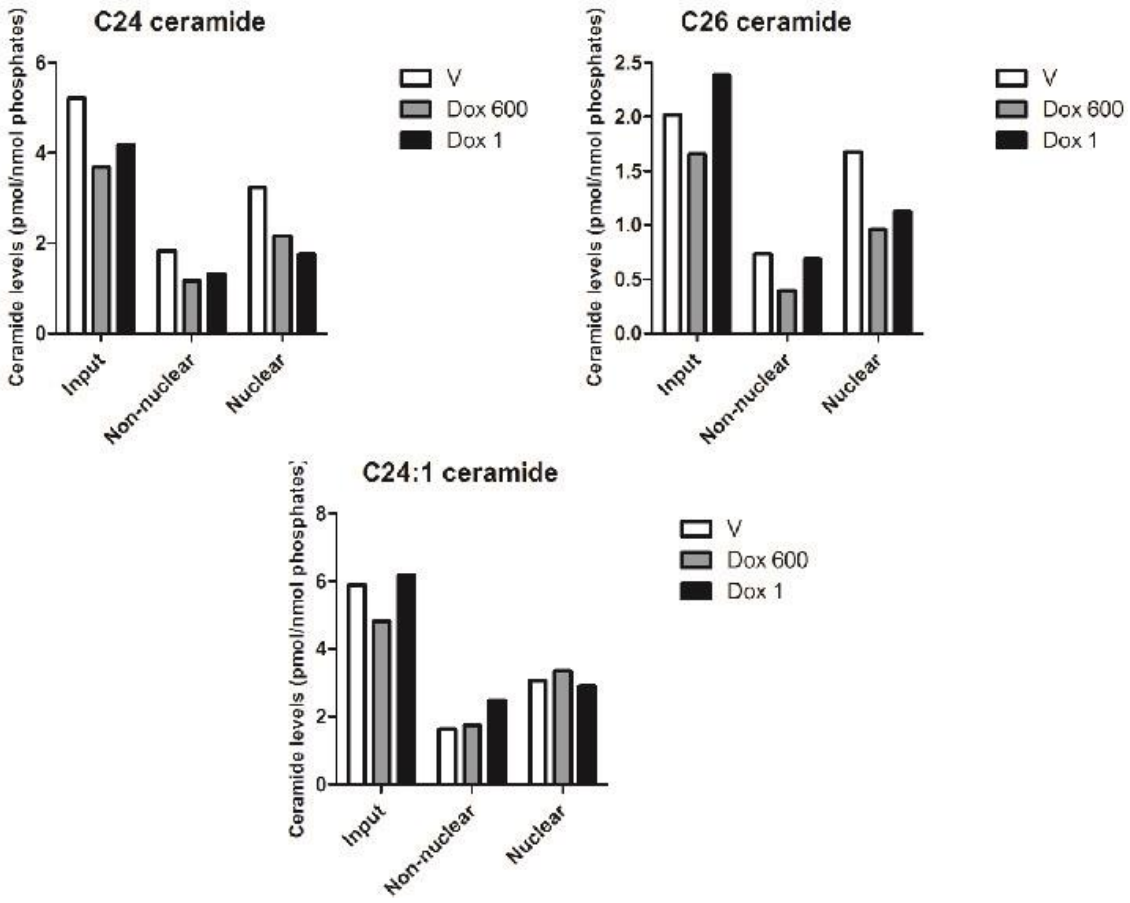


Figure S11: Ceramide species that show no changes

MCF-7 cells were plated in 100 mm dishes and were treated with either vehicle or different concentrations of doxorubicin for 24 hours. Following that lipids were extracted and send for analysis by LC/MS. Shown are the ceramide species with no significant changes.

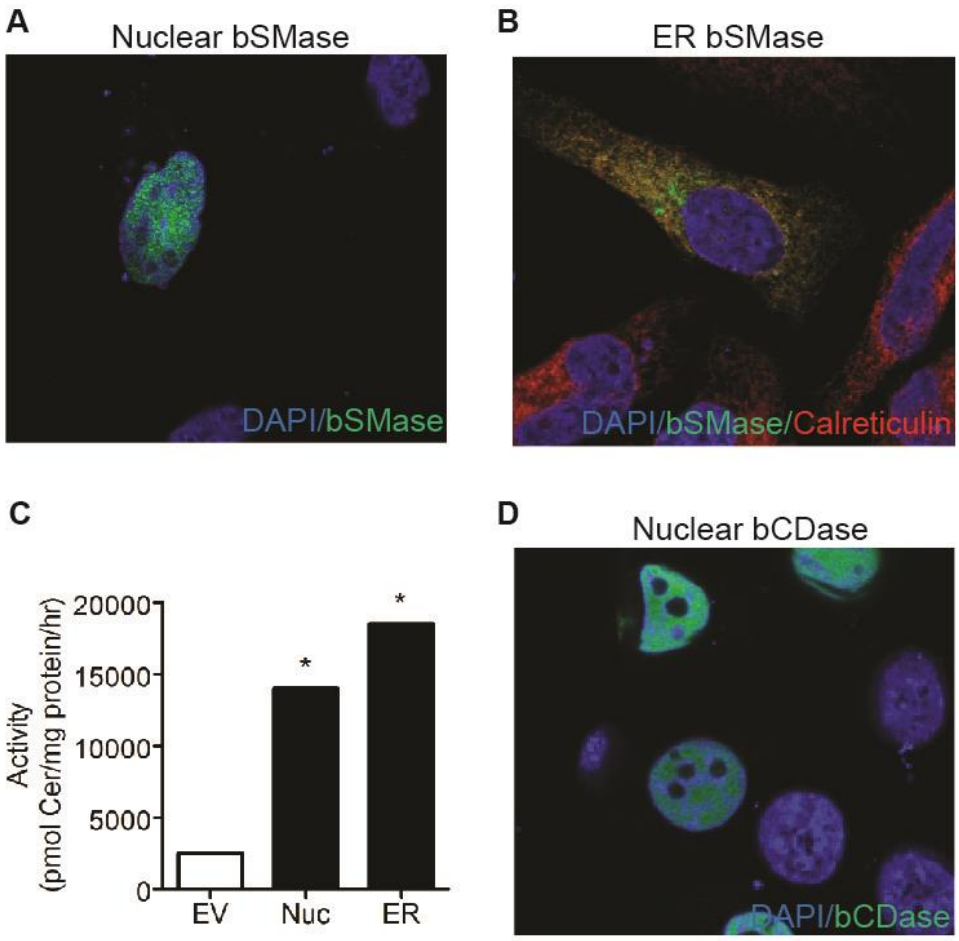


Figure S12: Verification of correct targeting of nuclear bacterial sphingomyelinase and nuclear bacterial ceramidase

(**A-B**) MCF-7 cells were plated in 35 mm confocal dishes and were transfected with Nuclear-bSMase or ER-bSMase for 24 hours after which fixed and immunofluorescence was done for V5, DAPI, and calreticulin. (**C**) MCF-7 cells were seeded in 60 mm dishes and were transfected with nuclear-bSMase (Nuc) or ER-bSMase (ER) for 24 hours after which they were lysed and NSMase activity assay was performed, * denotes values outside of the linear range of the assay. (**D**) MCF-7 cells were plated in 35 mm confocal dishes and were transfected with Nuclear-bCdase for 24 hours after which fixed and immunofluorescence was done for V5 and DAPI.

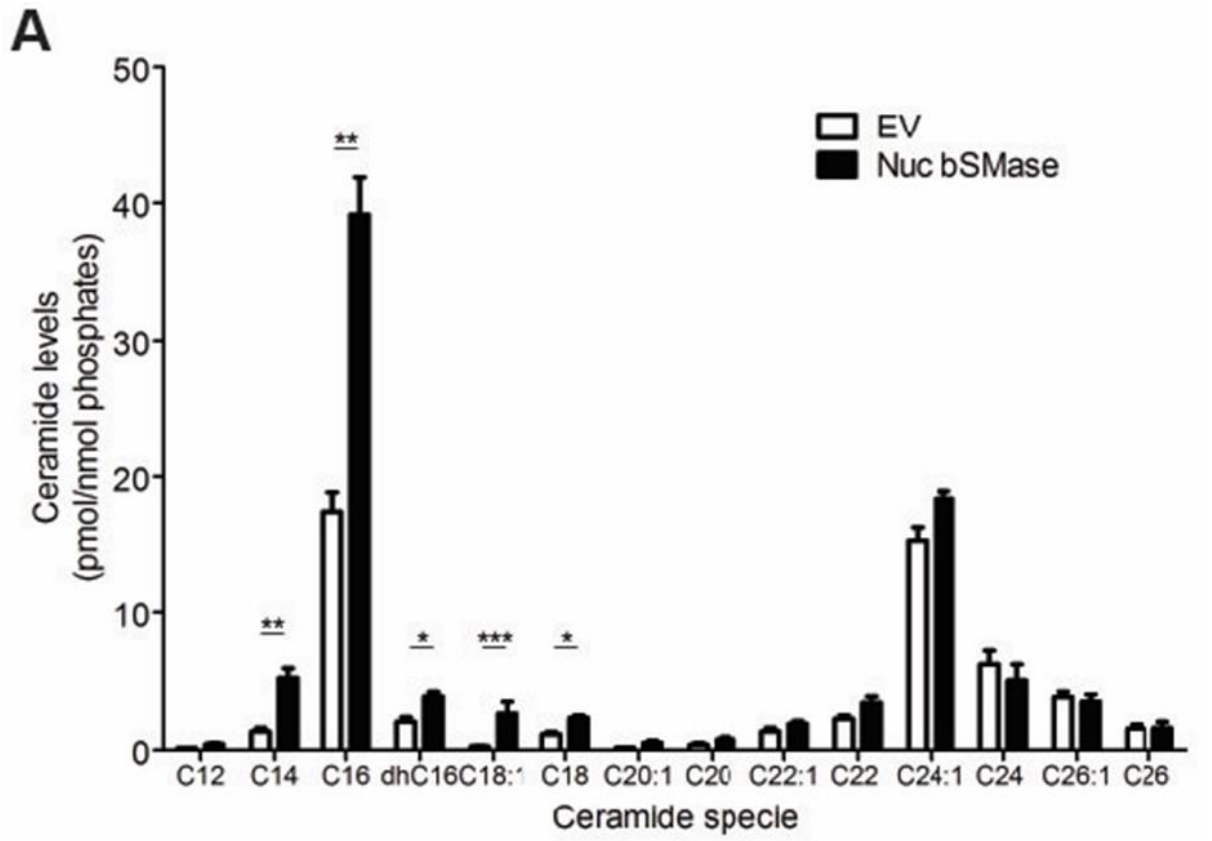


Figure S13 A: Lipid data after overexpression of nuclear bacterial sphingomyelinase

(**A**) MCF-7 cells were seeded in 60 mm dishes and either empty vector (EV) or nuclear bacterial sphingomyelinase (Nuc bSMase) were overexpressed for 24 hours. Cells were collected, lipids extracted and sent for LC/MS. Shown are the ceramide species changes, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

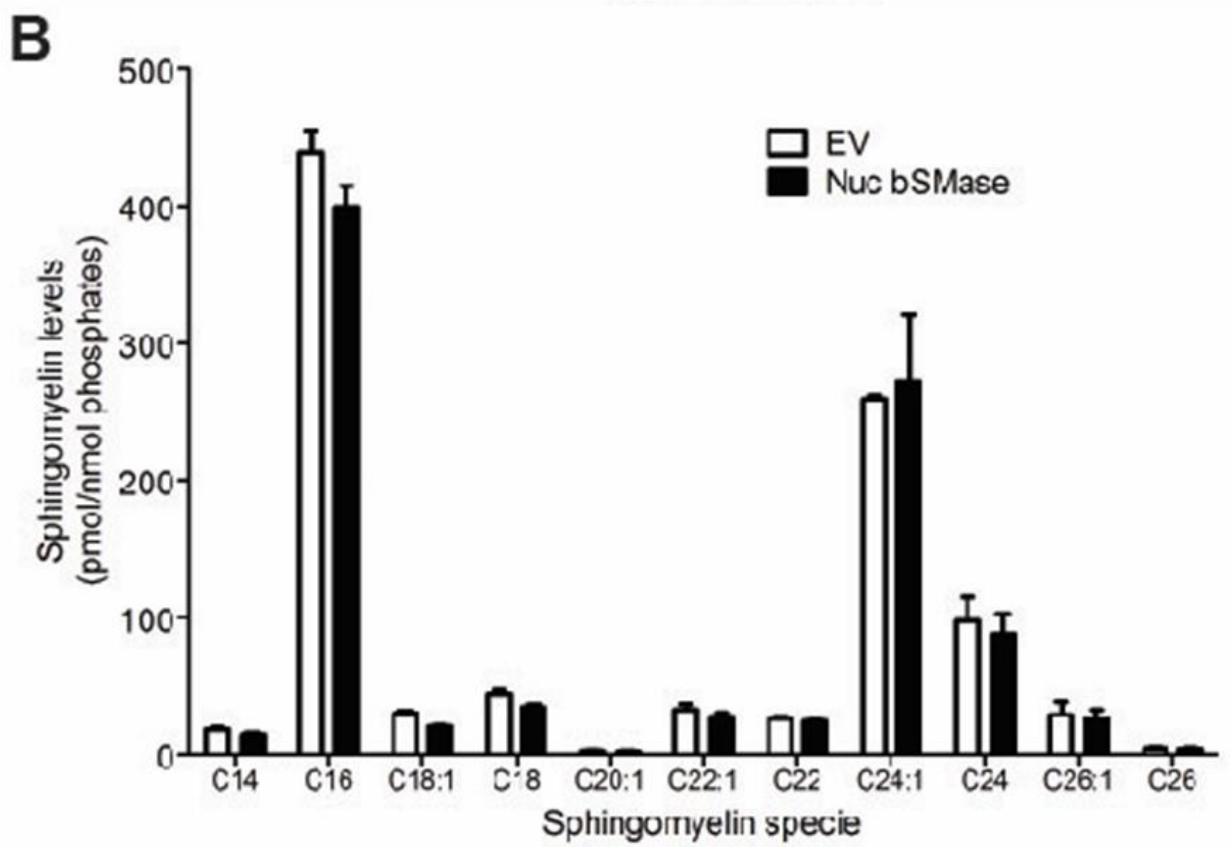


Figure S13 B: Lipid data after overexpression of nuclear bacterial sphingomyelinase

(**B**) MCF-7 cells were seeded in 60 mm dishes and either empty vector (EV) or nuclear bacterial sphingomyelinase (Nuc bSMase) were overexpressed for 24 hours. Cells were collected, lipids extracted and sent for LC/MS. Shown are the sphingomyelin species changes.

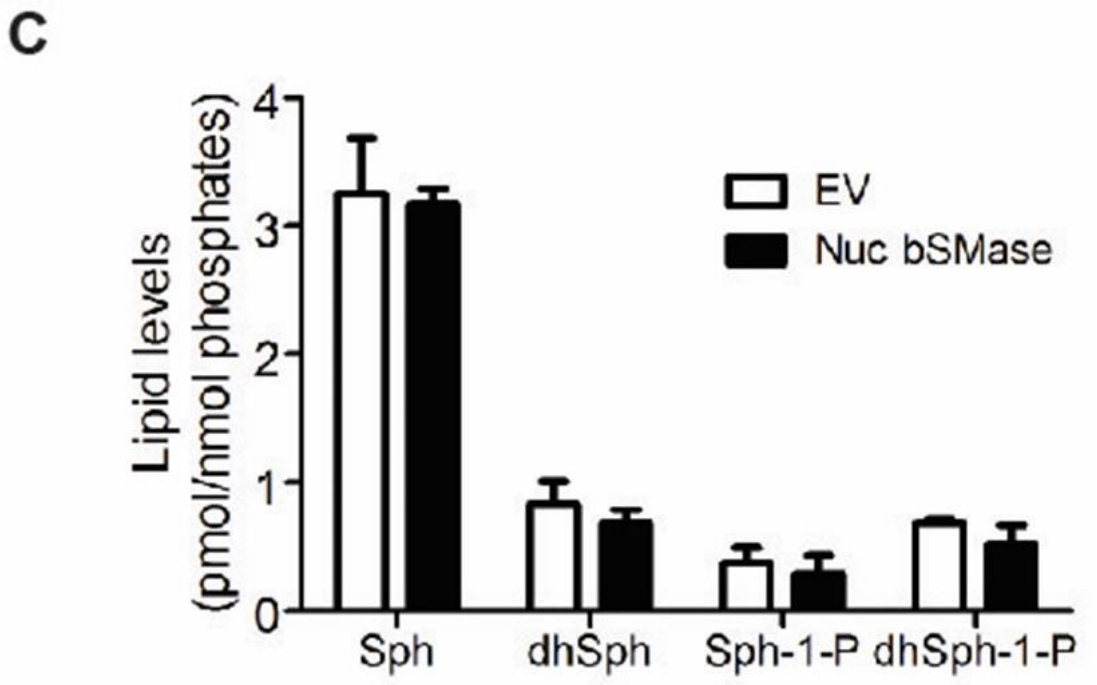


Figure S13 C: Lipid data after overexpression of nuclear bacterial sphingomyelinase

(**C**) MCF-7 cells were seeded in 60 mm dishes and either empty vector (EV) or nuclear bacterial sphingomyelinase (Nuc bSMase) were overexpressed for 24 hours. Cells were collected, lipids extracted and sent for LC/MS. Shown are the sphingosine (Sph), dihydrosphingosine (dhSph), sphingosine-1-phosphate (Sph-1-P) and the dihydrosphingosine-1-phosphate (dhSph-1-P) levels.

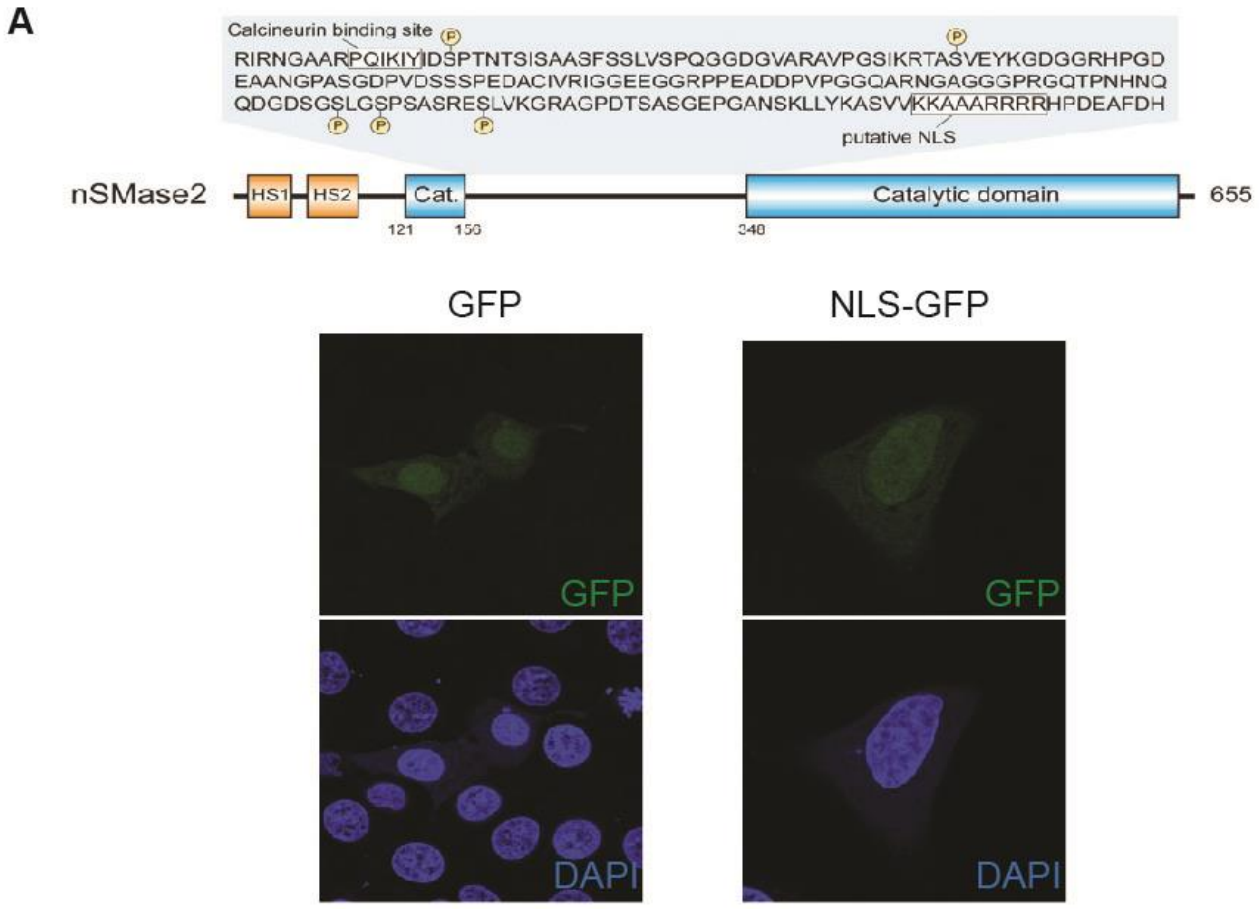


Figure S14 A: Studying the mechanism of localization of nSMase2 to the nucleus

(**A**) MCF-7 cells were plated in 35 mm confocal dishes and were transfected with GFP or NLS-GFP for 24 hours after which they were fixed and immunofluorescence was done for GFP and DAPI.

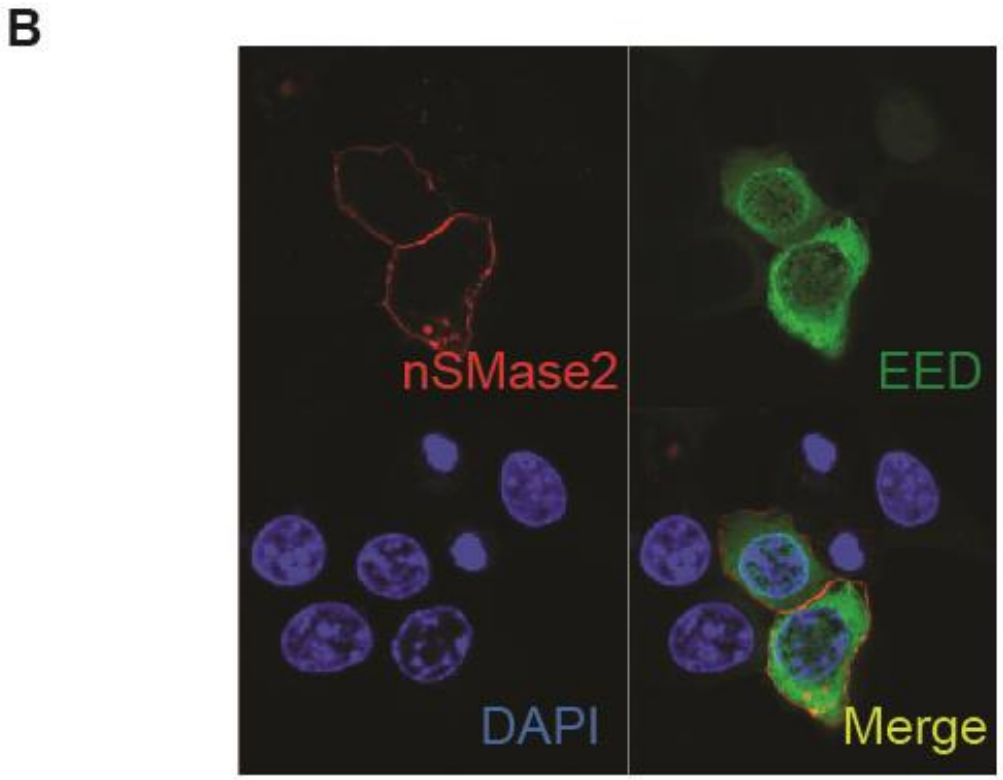


Figure S14 B: Studying the mechanism of localization of nSMase2 to the nucleus

(**B**) MCF-7 cells were plated in 35 mm confocal dishes and were transfected with nSMase2 and EED for 24 hours after which they were fixed and immunofluorescence was done for V5 (EED), His (nSMase2) and DAPI.

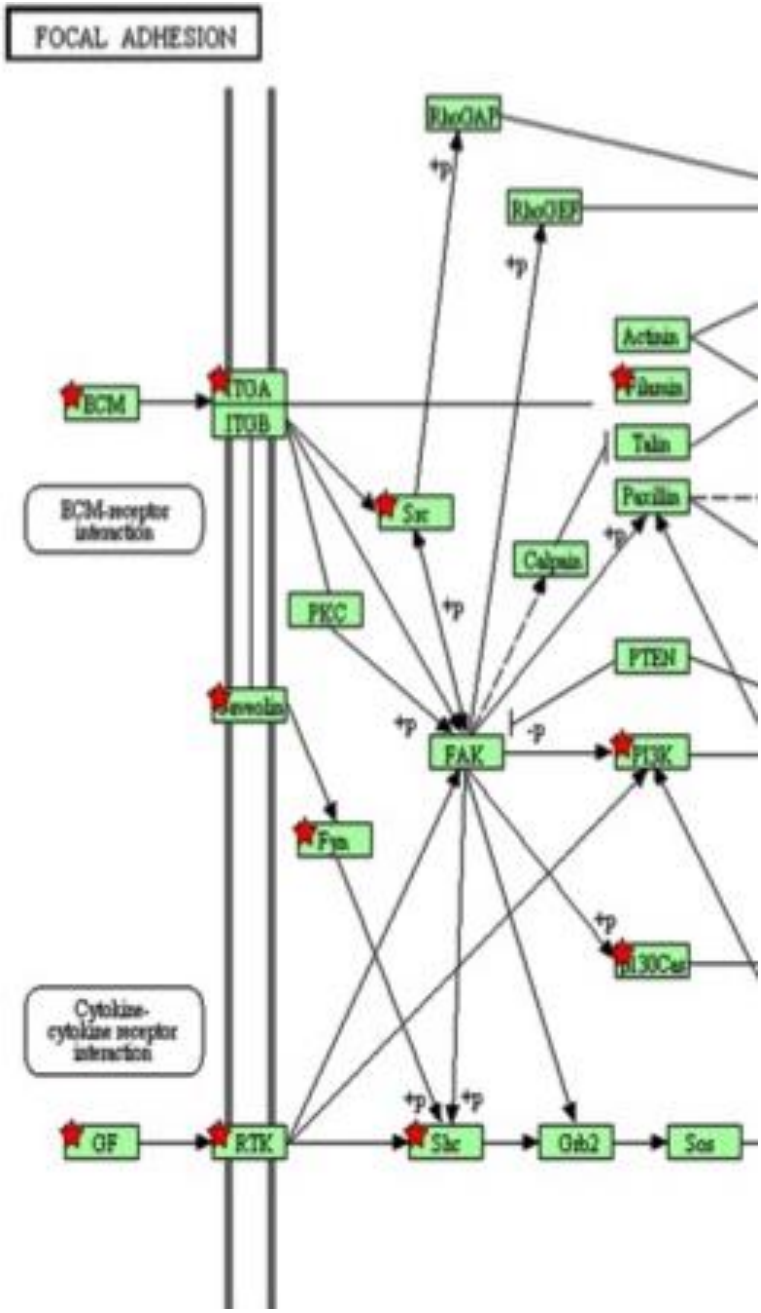


Figure S15: Kegg pathway representation of genes focal adhesion genes upregulated by doxorubicin

Shown in red stars are genes that are upregulated 2 folds or more by doxorubicin treatment in MCF-7 cells as per RNA sequencing data.

Tables

Table1: qRT PCR array result for sphingolipid enzymes changes with doxorubicin

Doxorubicin (nM)	200	400	600	800	1000
ASAH1	1.3996	-1.0105	-1.014	1.2527	1.8213
NAAA	1.459	2.0209	2.4453	4.0139	8.7241
ASAH2	-1.3426	2.0922	1.007	6.7039	11.5115
ASAH2B	1.4191	2.0069	1.9453	1.7231	-1.1134
ACER1	-2.8382	-1.0246	1.434	2.2423	3.9862
ACER2	22.2387	47.6697	14.9285	9.68	7.9723
ACER3	1.4093	1.9386	-1.6245	2.166	2.7798
CERK	-3.3058	-4.6751	-4.6913	-6.3423	-4.1554
CERKL	-1.429	-1.0105	1.5052	3.6175	3.9586
COL4A3BP	-1.4093	-2.0069	-1.2924	-1.1851	-1.6876
DEGS1	-2.7226	1.0175	1.0497	-1.429	-3.042
DEGS2	1.4191	3.9313	3.0525	1.6301	-1.2879
ELOVL6	-5.1515	-3.6427	-1.8661	-4.6428	-4.84
GALC	3.0631	4.0982	1.6133	4.6107	2.4538
SGPP1	1.3899	1.9793	1.9319	2.4368	1.4489
GBA	1.3899	1.3149	-1.5369	1.2184	1.3803
GBA2	-1.3996	2.0069	4.0278	-1.4191	-2.3054
GBA3	-1.4191	1.1688	1.0943	1.2184	4.1267
B4GALT6	-1.4191	-1.0035	-1.2658	-1.7715	-2.7038
CERS1	-2.7606	1.0035	-1.0644	1.3803	-1.0105
CERS2	-2.2268	-1.5746	-1.5911	-1.2879	-2.3376
CERS3	-1.2614	1.9656	-1.0644	3.5677	1.2016

CERS4	-1.4191	1.9656	-1.1567	-3.3519	-5.5597
CERS5	1.2968	2.3054	-1.1096	1.1212	-1.1527
CERS6	-2.858	1.9656	1.1096	1.1527	-2.1361
SGMS1	1.3708	1.0317	-1.0353	1.2016	-1.21
SGMS2	1.4093	5.4453	2.9485	2.4538	1.0389
SGPL1	-1.4489	1.8213	-2.2191	1.429	-1.8213
SGPP2	-1.5	-1.0681	-2.1435	-1.3149	-2.4033
SMPD1	2.7992	7.9173	4.2281	2.2268	-1.0389
SMPD2	-1.3519	1.8856	-1.0497	-1.6529	-3.4462
SMPD3	5.5983	70.2779	63.1189	9.8833	5.4453
SMPD4	-2.8382	-2.0209	1.4845	-1.676	-2.3867
GLTP	1.3996	3.9313	1.1329	-1.5856	-1.6189
PSAP	1.9656	3.0001	2.7702	1.7231	1.8213
SPHK1	1.3803	3.9862	2.8481	1.2879	1.2016
SPHK2	-1.4191	-1.0105	-1.9588	-6.0839	-8.6638
SPTLC1	-1.4191	1.9793	1.6133	1.4389	-1.3149
SPTLC2	-2.8186	-2.0069	-1.1567	-2.8979	-5.5213
SPTLC3	1.2614	1.9931	1.7532	4.3924	2.4033
UGCG	-4.8065	-2.0209	-1.3195	-5.716	-13.4078
GLA	-1.676	1.9931	1.6702	1.0246	1.0175
NPC1	1.0035	2.858	1.4142	-1.244	-2.3214
ACTB	-1.4093	-1.9931	-1.0867	-1.1527	-1.0461
GAPDH	1.4093	1.9931	1.0867	1.1527	1.0461

Table 2: Comparison betweem yeast and mammalian findings of Isc1 and nSMase2

	Isc1	nSMase2
Stimulus	Hydroxyurea	Doxorubicin
Upstream regulators	Bub1/Mad1/Kar3	ATR/Chk1/p53
Upstream mode of regulation	Post-transcriptional	Transcriptional
Localization	Nuclear	Nuclear
Lipid produced	C18:1 phytoceramide	C18:1 ceramide
Possible phosphatase activated	CDC55 (subunit of PP2A)	B55 (subunit of PP2A)
Phospho targets	Cohesin/Condensin protein complexes	Cohesin/Condensin protein complexes
Biology	Protective Stablizes microtubule in mitotic spindle checkpoint	Protective Mediates S phase prolongation after DNA damage

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